Bohn, Brent

From: Gibbons, Catherine
Sent: Friday, December 04, 2015 7:40 PM
To: Bohn, Brent
Subject: FW: Another Cr6 slide

From: Khan, Elaine@OEHHA [mailto:Elaine.Khan@oehha.ca.gov]
Sent: Tuesday, June 24, 2014 7:25 PM
To: Gibbons, Catherine <Gibbons.Catherine@epa.gov>
Subject: RE: Another Cr6 slide

It's late and I'm sure you're tired. Are you even planning on going home tonight?

From: Gibbons, Catherine [mailto:Gibbons.Catherine@epa.gov]
Sent: Tuesday, June 24, 2014 4:24 PM
To: Khan, Elaine@OEHHA
Subject: RE: Another Cr6 slide

LOL! As usual, turns out you have sent me this paper before and I forgot about it ☹ IN FACT, it was sitting RIGHT NEXT TO ME in a pile of "important papers to review" at this very second!

From: Khan, Elaine@OEHHA [mailto:Elaine.Khan@oehha.ca.gov]
Sent: Tuesday, June 24, 2014 7:16 PM
To: Gibbons, Catherine
Subject: RE: Another Cr6 slide

I notice slide 3 shows villous vacuolization – Chad had said before that they considered that part of the damage that led to crypt hyperplasia (because I noted that they had more animals with crypt hyperplasia than there were animals with apoptosis or villous atrophy) but I pointed out that vacuolization is not always a precursor of cell death. It's a stress response and can often lead to survival. See attached paper by Galluzzi et al. (highlights on p. 112 and Fig. 4 on p. 114).

Also, Ki-67 is a cell proliferation marker:

J Cell Physiol. 2000 Mar;182(3):311-22.

The Ki-67 protein: from the known and the unknown.

Scholzen T[1], Gerdes J.

Author information

- [1]Division of Molecular Immunology, Research Center Borstel, Germany. tscholzen@fz-borstel.de

Abstract

The expression of the human Ki-67 protein is strictly associated with cell proliferation. During interphase, the antigen can be exclusively detected within the nucleus, whereas in mitosis most of the protein is relocated to the surface of the chromosomes. The fact that the Ki-67 protein is present during all active phases of the cell cycle (G(1), S, G(2), and mitosis), but is absent from resting cells (G(0)), makes it an excellent marker for determining the so-called growth fraction

of a given cell population. In the first part of this study, the term proliferation marker is discussed and examples of the applications of anti-Ki-67 protein antibodies in diagnostics of human tumors are given. The fraction of Ki-67-positive tumor cells (the Ki-67 labeling index) is often correlated with the clinical course of the disease. The best-studied examples in this context are carcinomas of the prostate and the breast. For these types of tumors, the prognostic value for survival and tumor recurrence has repeatedly been proven in uni- and multivariate analysis. The preparation of new monoclonal antibodies that react with the Ki-67 equivalent protein from rodents now extends the use of the Ki-67 protein as a proliferation marker to laboratory animals that are routinely used in basic research. The second part of this review focuses on the biology of the Ki-67 protein. Our current knowledge of the Ki-67 gene and protein structure, mRNA splicing, expression, and cellular localization during the cell-division cycle is summarized and discussed. Although the Ki-67 protein is well characterized on the molecular level and extensively used as a proliferation marker, the functional significance still remains unclear. There are indications, however, that Ki-67 protein expression is an absolute requirement for progression through the cell-division cycle.

So I'm not sure if that's mouse or rat data he's showing in slide 4, but if it's mouse, there shouldn't be a decrease in Ki-67 at 91 days compared to 8 days.

From: Gibbons, Catherine [mailto:Gibbons.Catherine@epa.gov]
Sent: Tuesday, June 24, 2014 3:14 PM
To: Khan, Elaine@OEHHA
Subject: Another Cr6 slide

Ha! Check this out, he's mentioning the paper and studies pointing to a villus origin for tumors.

Bohn, Brent

From: Gibbons, Catherine
Sent: Friday, December 04, 2015 7:40 PM
To: Bohn, Brent
Subject: FW: Another Cr6 slide
Attachments: Scholzen 2000.pdf

From: Khan, Elaine@OEHHA [mailto:Elaine.Khan@oehha.ca.gov]
Sent: Tuesday, June 24, 2014 7:43 PM
To: Gibbons, Catherine <Gibbons.Catherine@epa.gov>
Subject: RE: Another Cr6 slide

Ok, I just checked and slide 4 in this attachment (Science Question 4 presentation) refers to the mouse data (Thompson et al. 2013, Fig. 4). This is what they say in the paper:
"In addition, transcript levels of
Ki67, a common marker of crypt cell proliferation (Itzkovitz
et al., 2012; Potten et al., 1997), were significantly elevated at
_170 mg/l SDD at both day 8 and day 91 (Figure 4H).
Together, these data suggest that Cr(VI) increased crypt
hyperplasia as early as one week of exposure to very high
concentrations in drinking water."

To me, the decrease in Ki-67 at 91 days looks very similar to the their figure in slide 2 of their Science Question 3 presentation, where they say that "effects in rats might have been subsiding..." Attached is the Ki-67 review article I cited below – I haven't read it yet, but we may not need to read it since they themselves are saying that it's a marker of crypt cell proliferation.

From: Gibbons, Catherine [mailto:Gibbons.Catherine@epa.gov]
Sent: Tuesday, June 24, 2014 4:24 PM
To: Khan, Elaine@OEHHA
Subject: RE: Another Cr6 slide

LOL! As usual, turns out you have sent me this paper before and I forgot about it ☹ IN FACT, it was sitting RIGHT NEXT TO ME in a pile of "important papers to review" at this very second!

From: Khan, Elaine@OEHHA [mailto:Elaine.Khan@oehha.ca.gov]
Sent: Tuesday, June 24, 2014 7:16 PM
To: Gibbons, Catherine
Subject: RE: Another Cr6 slide

I notice slide 3 shows villous vacuolization – Chad had said before that they considered that part of the damage that led to crypt hyperplasia (because I noted that they had more animals with crypt hyperplasia than there were animals with apoptosis or villous atrophy) but I pointed out that vacuolization is not always a precursor of cell death. It's a stress response and can often lead to survival. See attached paper by Galluzzi et al. (highlights on p. 112 and Fig. 4 on p. 114).

Also, Ki-67 is a cell proliferation marker:

J Cell Physiol. 2000 Mar;182(3):311-22.

The Ki-67 protein: from the known and the unknown.

Scholzen T[1], Gerdes J.

Author information

- [1]Division of Molecular Immunology, Research Center Borstel, Germany. tscholzen@fz-borstel.de

Abstract

The expression of the human Ki-67 protein is strictly associated with cell proliferation. During interphase, the antigen can be exclusively detected within the nucleus, whereas in mitosis most of the protein is relocated to the surface of the chromosomes. The fact that the Ki-67 protein is present during all active phases of the cell cycle (G(1), S, G(2), and mitosis), but is absent from resting cells (G(0)), makes it an excellent marker for determining the so-called growth fraction of a given cell population. In the first part of this study, the term proliferation marker is discussed and examples of the applications of anti-Ki-67 protein antibodies in diagnostics of human tumors are given. The fraction of Ki-67-positive tumor cells (the Ki-67 labeling index) is often correlated with the clinical course of the disease. The best-studied examples in this context are carcinomas of the prostate and the breast. For these types of tumors, the prognostic value for survival and tumor recurrence has repeatedly been proven in uni- and multivariate analysis. The preparation of new monoclonal antibodies that react with the Ki-67 equivalent protein from rodents now extends the use of the Ki-67 protein as a proliferation marker to laboratory animals that are routinely used in basic research. The second part of this review focuses on the biology of the Ki-67 protein. Our current knowledge of the Ki-67 gene and protein structure, mRNA splicing, expression, and cellular localization during the cell-division cycle is summarized and discussed. Although the Ki-67 protein is well characterized on the molecular level and extensively used as a proliferation marker, the functional significance still remains unclear. There are indications, however, that Ki-67 protein expression is an absolute requirement for progression through the cell-division cycle.

So I'm not sure if that's mouse or rat data he's showing in slide 4, but if it's mouse, there shouldn't be a decrease in Ki-67 at 91 days compared to 8 days.

From: Gibbons, Catherine [mailto:Gibbons.Catherine@epa.gov]
Sent: Tuesday, June 24, 2014 3:14 PM
To: Khan, Elaine@OEHHA
Subject: Another Cr6 slide

Ha! Check this out, he's mentioning the paper and studies pointing to a villus origin for tumors.

JOURNAL OF CELLULAR PHYSIOLOGY 182:311–322 (2000)

REVIEW ARTICLES

The Ki-67 Protein: From the Known and the Unknown

THOMAS SCHOLZEN* AND JOHANNES GERDES
Division of Molecular Immunology, Research Center Borstel, Germany

The expression of the human Ki-67 protein is strictly associated with cell proliferation. During interphase, the antigen can be exclusively detected within the nucleus, whereas in mitosis most of the protein is relocated to the surface of the chromosomes. The fact that the Ki-67 protein is present during all active phases of the cell cycle (G_1, S, G_2, and mitosis), but is absent from resting cells (G_0), makes it an excellent marker for determining the so-called growth fraction of a given cell population. In the first part of this study, the term *proliferation marker* is discussed and examples of the applications of anti-Ki-67 protein antibodies in diagnostics of human tumors are given. The fraction of Ki-67-positive tumor cells (the Ki-67 labeling index) is often correlated with the clinical course of the disease. The best-studied examples in this context are carcinomas of the prostate and the breast. For these types of tumors, the prognostic value for survival and tumor recurrence has repeatedly been proven in uni- and multivariate analysis. The preparation of new monoclonal antibodies that react with the Ki-67 equivalent protein from rodents now extends the use of the Ki-67 protein as a proliferation marker to laboratory animals that are routinely used in basic research. The second part of this review focuses on the biology of the Ki-67 protein. Our current knowledge of the Ki-67 gene and protein structure, mRNA splicing, expression, and cellular localization during the cell-division cycle is summarized and discussed. Although the Ki-67 protein is well characterized on the molecular level and extensively used as a proliferation marker, the functional significance still remains unclear. There are indications, however, that Ki-67 protein expression is an absolute requirement for progression through the cell-division cycle. J. Cell. Physiol. 182:311–322, 2000. © 2000 Wiley-Liss, Inc.

With regard to the Ki-67 protein, the scientific community can be divided into three groups. The first group, that is, for those working in pathology, the Ki-67 protein and the corresponding antibodies are well known, whereas a second group of researchers working in other fields often tend to ignore this protein. The third, and by far the smallest group, consists of those who are actually interested in the biology and function of the Ki-67 protein itself. The aim of this review is to give information about the properties of the Ki-67 protein and its use as a proliferation marker in research and diagnostics. Moreover, the authors hope that this study will also help to raise more interest in the biology and the unique features of the Ki-67 protein.

The Ki-67 protein was originally defined by the prototype monoclonal antibody Ki-67 (Gerdes et al., 1983), which was generated by immunizing mice with nuclei of the Hodgkin lymphoma cell line L428. The name is derived from the city of origin (Kiel) and the number of the original clone in the 96-well plate. Because the antigen was not initially characterized, it was referred to mainly as the *Ki-67 antigen.* When the antigen was found to be a protein and the primary structure could be deduced from the corresponding cDNA, it revealed no homology to any known polypeptide (see later discussion). For this reason and because the function of the protein remained indistinct, the initial name Ki-67 was kept. To prevent confusion, we refer to the prototype antibody and the antigen as the Ki-67 antibody and Ki-67 protein, respectively, throughout this study.

Characterization of the Ki-67 antibody revealed an interesting staining pattern. The antibody was reactive with a nuclear structure present exclusively in proliferating cells. A detailed cell cycle analysis revealed that the antigen was present in the nuclei of cells in the G_1, S, and G_2 phases of the cell division cycle as well as in mitosis. Quiescent or resting cells in the G_0 phase did not express the Ki-67 antigen (Gerdes et al., 1984). Because the Ki-67 antigen was present in all proliferating cells (normal and tumor cells), it soon became evident that the presence of this structure is an excellent operational marker to determine the growth fraction of a given cell population. For this reason, antibodies against the Ki-67 protein were increasingly used as diagnostic tools in different types of neoplasms. Despite this broad application, the nature of the antigen

*Correspondence to: Thomas Scholzen, Division of Molecular Immunology, Research Center Borstel, Parkallee 22, D-23845 Borstel, Germany. E-mail: tscholzen@fz-borstel.de

Received 11 August 1999; Accepted 11 August 1999

remained unclear until it was identified as a protein by screening a cDNA expression library (Gerdes et al., 1991). The complete primary structure was published two years later after cloning and sequencing the entire cDNA (Schlüter et al., 1993). Two protein isoforms were originally described, generated by alternative splicing of an mRNA precursor transcribed from a single gene. Both isoforms with calculated molecular masses of 320 and 359 kD contain a large central region with 16 repetitive elements (Ki-67 repeats) encoded by a single exon of 6845 base pairs. Meanwhile, the entire gene locus of the Ki-67 protein has been sequenced as well, comprising almost 30,000 base pairs (Duchrow et al., 1996).

In 1983 when the first monoclonal antibody against the Ki-67 protein was described, a picture of the molecular mechanisms of the eukaryotic cell cycle control was just beginning to develop. In the same year, the term *cyclin* was proposed for proteins that are destroyed at certain points in the cell cycle (Evans et al., 1983). In the following years, rapid progress was made in the elucidation of the intricate network of cyclins, cyclin-dependent kinases, and their inhibitors that govern the meticulously coordinated cell cycle progression (for review see Graña and Reddy, 1995; Pines, 1995; Lew and Kornbluth, 1996; Arellano and Moreno, 1997). Advancement has also been made in understanding the factors that control key events in the cell division cycle, for example, the transition from one phase to the next, DNA replication, and chromosome condensation and segregation. The tumor supressor genes p53 and pRb have been shown to be major targets in the control of the transition from G_1 to S phase (reviewed in Paggi et al., 1996; Levine, 1997; Mayol and Graña, 1997; Agarwal et al., 1998; Graña et al., 1998; Stiegler et al., 1998). Furthermore, the role of the ORC (origin recognition complex) and Mcm (mini chromosome maintenance) proteins in the control of DNA replication have been described (for review see Kearsey and Labib, 1998; Tada and Blow, 1998). Although our knowledge of the molecular regulation of the cell division cycle is far from being complete, a vast body of data has been accumulated and is beginning to form into a detailed picture of a complex network that is regulated by expression, modification (e.g., phosphorylation, ubiquitination), degradation, and translocation of a number of key proteins. Despite this extensive knowledge, none of these key regulators has yet been functionally linked to the Ki-67 protein. This is striking, because as mentioned earlier, Ki-67 protein expression is strictly correlated to cell proliferation and to the active phases of the cell cycle. To our knowledge no other protein shows an expression pattern that is equally tightly regulated in dependence to the proliferative status of the cell. One would therefore expect that the role of the Ki-67 protein in cell proliferation would be fundamental and therefore easy to reveal. If the former expectation may still hold to be true, the latter has definitely been proven wrong during the last 16 years.

Ki-67 EXPRESSION AS A MARKER FOR PROLIFERATING CELLS

Requirements for a proliferation marker

Before discussing the use of the Ki-67 protein in tumor diagnostics, it may be worth having a more theoretical look at the term *proliferation marker* in this context. The expression of the Ki-67 protein is thought to be an indicator for cells within the growth fraction of a given population, that is, the fraction of cells born into the proliferative category (Alison, 1995). For this kind of marker two requirements have been postulated (van Dierendonck et al., 1989): (1) the antigen should be continuously present during the cell cycle of all cell types and (2) the transition to whatever type of nonproliferative state from any part of the cell cycle should be followed by a rapid disappearance of the antigen. Although it has been reported that Ki-67 antigen staining may be faint or even undetectable at the onset of DNA synthesis (see later discussion), it is generally accepted that the Ki-67 protein is expressed during all active phases of the cell cycle. Furthermore, all tissues tested showed Ki-67 staining in cell populations known to proliferate (Gerdes et al., 1983) and no convincing evidence has been presented that the Ki-67 protein may not be expressed by any proliferating human cell type.

The second requirement is more difficult to fulfill. One problem is the definition of the term *nonproliferative state.* This expression presupposes that a cell can be found in only two clearly defined states: the proliferative or the nonproliferative. In practice, cells tend to have a rather broad range of intermitotic times. Intermitotic times can vary greatly, especially in vivo and under suboptimal growth conditions in vitro. Because there is no unequivocal feature that can distinguish between "proliferative" and "nonproliferative" cells, it is always a matter of definition which cells are termed nonproliferative. Different techniques have been developed to access cell proliferation (for review, see Alison, 1995). Standard procedures to determine the growth fraction are generally based on experimental protocols, in which newly synthesized DNA is labeled during the S phase. Samples are taken at different time points and the fraction of labeled nuclei (or labeled mitoses) is estimated. The data obtained in such experiments, together with assumptions about cell proliferation kinetics, are subsequently used to estimate a theoretical value for the growth fraction of the population. One has to consider that these approaches will underestimate the growth fraction if a considerable fraction of cells has intermitotic times that exceed the labeling interval. In contrast, Ki-67 protein expression directly reflects a certain physiological state of the cell. Although the functional role of the Ki-67 protein during cell proliferation is unknown, it is unquestionable that Ki-67 protein expression and cell proliferation are closely linked.

Even if an "ideal" proliferation marker could be found, there are theoretical limitations for its use in estimating the growth fraction. As mentioned previously, such an ideal proliferation marker must label "proliferative" cells during all (active) phases of the cell cycle. A cell should be positive as long as it is going to divide. The problem is that the term *going to divide* refers not to the actual state of the cell but to an event in the future. The cell must make the final decision whether to divide at some time point during the cell cycle. Let us assume, for example, that the decision is made in mid-G_1 phase. As stated earlier, for an ideal proliferation marker, one would postulate that during

all cell cycle phases, including early G_1 phase, the cell should be positive if the cell is going to divide, but negative if the cell is going to become quiescent or terminally differentiated. This leads to the contradiction that the proliferation marker must indicate the decision of the cell *before* it has actually been made. A correct indication of the proliferative state can be made only if the decision whether to continue proliferation is made before or at least at the time of the previous cell division. This assumption, however, is in disagreement with experimental data proving that the decision can be made in later phases of the cell cycle. Some authors postulate that cells can enter a quiescent state even after completing DNA synthesis (Darzynkiewicz and Traganos, 1982; Drewinko et al., 1984; Lazebnik et al., 1991; Wei et al., 1993). This leads to the conclusion that a proliferation marker can be used only to indicate the potential of a certain cell to divide, but not to predict the actual division of this cell.

This is especially true if the growth kinetics of a cell population is disturbed, for example, by administration of drugs. Van Oijen et al. (1998) showed that cells remain Ki-67 positive when DNA synthesis is blocked by hydroxyurea; the cells are arrested in mitosis by nocodazole or blocked in the cell cycle progression by the heterologous expression of the cyclin-dependent kinase inhibitor p21. They further demonstrated Ki-67 positive cells 24 h after inflicting DNA damage by the application of camptothecin. Under these conditions, p21 and p53 were upregulated and progression through the cell division cycle arrested as measured by incorporation of the thymidine analogue bromodeoxyuridine (BrdU). From these observations, the authors conclude that Ki-67 expression is not necessarily linked to actively proliferating cells and advise against the use of Ki-67 in studies of cells that overexpress p53 or p21. These statements result from the oversimplified model that cell proliferation can be assessed by monitoring some kind of master switch that, in any case, precisely reflects the proliferative state of the cell. In contrast to this model, all evidence supports the idea that cell proliferation is governed by a complex and intricate regulatory network. Artificially interfering with one part of the proliferative process will not necessarily lead to the corresponding adjustment of all involved parameters. In other words, it cannot be expected that a cell downregulates its Ki-67 protein expression just because DNA synthesis is blocked by the application of a drug. A cell that continues to express the Ki-67 protein is likely to maintain the potential to proliferate and may eventually do so after the block is released. In the case of experimentally inflicted DNA damage, Ki-67 antigen expression may also correspond to the ability of the cell to resume the cell division cycle after the DNA damage is repaired. The advice not to use Ki-67 in studies of cells that naturally overexpress p53 or p21 should be given only if it can be proven that such cells remain Ki-67 positive without proliferative activity for a prolonged time span. Up to now, convincing evidence for this is missing.

In conclusion, although the Ki-67 protein expression may not correspond in every case to the theoretically defined term of *growth fraction*, in general one finds a close correlation between these parameters. However, it should be kept in mind that positivity for the Ki-67 antigen does not necessarily mean that a cell is unquestionably going to divide. Such a cell may still decide to leave the active cell cycle and enter a quiescent state. It is also possible to arrest cells in the cell cycle without immediately influencing Ki-67 expression. In this context it has to be considered that positivity for the Ki-67 antigen may reflect the ability of a cell to continue to proliferate after the block is removed. In practice, these considerations should not be regarded as drawbacks. Because the Ki-67 index (percentage of cells staining positive for the Ki-67 antigen) is directly based on a physiological parameter involved in cell proliferation, it may give an even better insight into the growth characteristics of a tumor, its susceptibility to certain drugs, and to the outcome of a patient than the estimation of the growth fraction that precisely follows a theoretical definition. The usefulness of a marker in tumor diagnostics has to be tested for each tumor type and application. Only those markers that have been proven to be useful in practice should be considered. The prognostic value of the Ki-67 index has been established in numerous publications (see next section). But it is also evident that estimating the growth fraction alone is not sufficient to describe tumor growth. For example, the growth fraction (and the Ki-67 labeling index) relates only to the number (or fraction) of proliferative cells but not to the time needed for the completion of an intermitotic cycle. In other words, the estimation of the growth fraction gives information only about the state but not about the rate of proliferation; therefore, an additional marker would be helpful to assess this parameter. In the future, multiparameter analysis may provide a better means of analyzing cell proliferation and tumor growth. This may not only improve the prognostic value, but may also be a prerequisite for choosing the appropriate type of therapy for each individual case.

Antibodies against the human Ki-67 protein: operational markers to estimate the growth fraction in malignant neoplasia

Since Gerdes et al. (1984) first suggested that the growth fraction as estimated by the Ki-67 labeling index could by of prognostic value in non-Hodgkin's lymphomas, numerous studies have been performed to examine the usefulness of this marker in various types of malignant neoplasms. It is out of the scope of this review to give a comprehensive overview about the use of antibodies against the Ki-67 protein in tumor diagnostics. To date, the *Medline database* lists nearly 4000 entries referring to the Ki-67 protein or anti-Ki-67 protein antibodies, and most of these publications deal with the prognostic value of the Ki-67 antigen (PubMed database August 1999, National Library of Medicine, Bethesda, MD 20894, USA). After it was shown that the Ki-67 protein could be detected during all active phases of the cell cycle but not in resting cells (Gerdes et al., 1983, 1984), it became evident that the Ki-67 antibody could be used as a tool to estimate the growth fraction of any human cell population. This is of special interest in tumor diagnostics, where the proliferative activity of a given tumor is often difficult to assess. Methods that are widely used in experimental research such as the determination of the cellular DNA content by flow cytometry or the in vitro or in vivo labeling of

newly synthesized DNA, are for practical and sometimes also ethical reasons difficult to apply to human tumors. In contrast, Ki-67 immunostaining can easily be performed on various types of cytological and histological preparations, such as smear, squash, cytocentrifuge preparations, and histological sections. In comparison to the counting of mitotic figures, the Ki-67 labeling index is more sensitive, because cells in all active phases of the cell cycle are recognized. Furthermore, the reliable assessment of mitotic figures needs more experience and is more time-consuming than the counting of immunohistologically stained nuclei. One major drawback of the original Ki-67 antibody was the fact that it could not be used in formalin-fixed paraffin sections, which are routinely used in histopathology. This disadvantage could be overcome by the preparation of the Ki-67 equivalent monoclonal antibody MIB-1, which can be used on paraffin sections after antigen reassessment by microwave-processing (Cattoretti et al., 1992).

Although the usefulness of the Ki-67 labeling index in tumor diagnostics has often been questioned, in recent years a growing number of studies have proven its prognostic value. This discussion has repeatedly led to the publication of editorials in reputed journals, arguing for the use of the Ki-67 labeling index in histopathology (Sawhney and Hall, 1992; Schwarting, 1993; Lloyd, 1998). One reason for the reluctance of many pathologists to use the Ki-67 labeling index was the competition with another "proliferation marker," the proliferating cell nuclear antigen (PCNA). Before the MIB-1 antibody was available, the prototype PCNA antibody PC10 had the advantage that it could be used in formalin-fixed paraffin sections. However, it could be shown that PC10 staining is very sensitive to fixation and that the antigen reassessment by microwave treatment led to the staining of quiescent cells (Schwarting, 1993; Kubbutat et al., 1994). This was confirmed by the finding that PCNA not only has a role in DNA replication, but is also involved in DNA repair. These multiple roles of PCNA (reviewed by Prosperi, 1997) strongly argue against the use of PCNA expression as a reliable marker for proliferating cells.

Meanwhile, the usefulness of the Ki-67 labeling index has been well established for various types of malignant neoplasms. In the case of multiple myeloma it could be shown that Ki-67 expression correlates with the course of the disease and, furthermore, is a useful marker in distinguishing multiple myeloma from monoclonal gammopathy of unknown significance (Drach et al., 1992; Miguel-Garcia et al., 1995). For soft-tissue sarcoma, the Ki-67 index is a significant predictor for overall survival of the patients as well as for the occurrence of distant metastasis (Ueda et al., 1989; Rudolph et al., 1997; Heslin et al., 1998; Huuhtanen et al., 1999).

The prognostic value of the Ki-67 index can be of particular importance in those types of cancers in which the clinical course is difficult to predict by histological criteria alone. A good example is prostate cancer, in which the predictive value of the Ki-67 labeling index has been studied in numerous publications. In multivariate analysis, it was found that the Ki-67 labeling index is an independent and significant prognostic factor for disease-specific survival if all stage and grade categories are included (Aaltomaa et al., 1997; Borre et al., 1998). Furthermore, for patients treated with radical prostatectomy Ki-67 expression is an independent predictor of disease recurrence and progression (Bettencourt et al., 1996; Bubendorf et al., 1996; Moul et al., 1996; Keshgegian et al., 1998; Bai et al., 1999). Similar results were obtained for patients treated with transurethral resection (Stattin et al., 1997). Moreover, the Ki-67 labeling index is also a significant predictive marker for the postradiation recurrence in patients with adenocarcinomas (Scalzo et al., 1998). The estimation of the Ki-67 labeling index may also be useful in lymphatic metastases from prostate cancer. In a study with patients who underwent pelvic lymphadenoectomy and ^{125}I implantation, a correlation was found between the fraction of Ki-67-positive malignant cells and the survival of patients (Cher et al., 1996). In the future, the pretherapeutic assessment of Ki-67 expression may become of increasing importance in the evaluation of tumor aggressiveness and the selection of adequate treatment. In a recent study, Bubendorf et al. (1998) assessed the p53, Bcl-2, and Ki-67 expression of prostate tumors in core needle biopsies. It could be shown that in multiparametric analysis, the Ki-67 labeling index was the only independent predictor of tumor-specific survival.

Another type of cancer in which the Ki-67 labeling index has been extensively studied is the breast carcinoma. During recent years a vast body of evidence has accumulated, which supports the concept that the Ki-67 labeling index is an independent prognostic factor for survival and tumor recurrence. Some of the more recent publications are summarized in Table 1. Together, these studies cover more than 4600 cases proving that the Ki-67 labeling index is a significant prognostic factor. This was found not only in univarite analysis but (when performed) most studies showed that Ki-67 is also of independent prognostic value in multivariate analysis.

The data summarized previously clearly argue for the assessment of the Ki-67 labeling index in tumor diagnostics. In this context it is worth mentioning that the diagnostic value of the Ki-67 index depends on the tumor type and the availability of protocols for treatment. For certain tumor types, no correlation could be demonstrated between prognosis and the Ki-67 labeling index. This does not necessarily mean that Ki-67 expression is not a good marker for proliferative activity; it merely indicates that there is no variability in the course of the disease in relation to the proliferative activity. It is obvious that the proliferative activity, as assessed by the Ki-67 labeling index, may be of importance in those types of treatment protocols that are aimed at dividing cells. Here the Ki-67 labeling index may be of great value in predicting how a tumor responds to a certain type of therapy. Consequently, the assessment of Ki-67 protein expression may become one of the crucial parameters for deciding which therapy is best for an individual tumor. We are now at the brink of the introduction of the Ki-67 labeling index into routine clinical diagnostics. For this purpose, however, there is an urgent need for an international standardization of the staining procedures and a clinical-pathological validation by randomized, multicenter prospective studies.

TABLE 1. Prognostic value of the Ki-67 labeling index in breast cancer

Reference	Prognostic significance[1]	Characteristics of studied group[1]	Antibody	Number of cases
Archer et al., 1995	OS (UA)	Grades II & III	Ki-67	92
Molino et al., 1997	OS, DFS (UA, MA)		Ki-67	322
Weikel et al., 1995	OS, DFS (UA)		Ki-67	549
Rudolph et al., 1999	OS, DFS (UA, MA)	N_0	Ki-S5	356
Clahsen et al., 1998	DFS (UA, MA)	pm, N_0	MIB-1	441
Dettmar et al., 1997	DFS (UA)	N_0	MIB-1	90
Domagala et al., 1996	OS (UA, MA)		MIB-1	186
Haerslev et al., 1996	OS (UA)		MIB-1	487
Jansen et al., 1998	OS, DFS (UA, MA)		MIB-1	341
Jensen et al., 1995	OS (UA, MA)	T_{1-2}, N_0, M_0	MIB-1	118
Keshgegian & Cnaan, 1995	DFS (UA)		MIB-1[2]	135
Pinder et al., 1995	OS (UA, MA)	tumor $\leq$ 5 cm	MIB-1	177
Rozan et al., 1998	DFS (UA); OS (UA, MA)		MIB-1	326
Seshadri et al., 1996	OS, DFS (UA, MA)	N_0, N_+	MIB-1	740
Veronese et al., 1996	OS, DFS (UA)		MIB-1[2]	246

[1]OS, overall survival; DFS, disease-free survival; UA, univariate analysis; MA, multivariate analysis; N_0, tumors from patients with negative axillary lymph nodes; N_+, tumors from patients with positive axillary lymph nodes; M_0, tumors from patients with no histological evidence of invasion of skin or deep fascia; T_{1-2}, tumor diameter $\leq$ 50 mm; pm, tumors from premenopausal patients.
[2]In addition, the labeling index was determined using the Ki-67 prototype antibody.

New applications for antibodies against the Ki-67 protein

A drawback to the application of Ki-67 antibodies in basic research is the fact that monoclonal antibodies raised against the human Ki-67 protein have often a rather limited cross-species reactivity. The prototype Ki-67 antibody detects the human protein as well as the Ki-67 equivalents from other primate species. A panel of new monoclonal antibodies against the Ki-67 protein was raised and designated *MIB* (for Molecular Immunology Borstel). Additionally, MIB-1 can be used to stain the Ki-67 equivalent proteins from various mammals (e.g., cattle, dog, horse, and sheep). Until recently, MIB-5 was the only monoclonal antibody reactive with the Ki-67 equivalents from rodents. Gerlach et al. (1997a,b) showed that MIB-5 is useful for the assessment of proliferating cells in rats. Employing immunohistochemical methods on tissue sections, the authors could demonstrate that it is possible to distinguish proliferative from nonproliferative cells during embryonic development. Moreover, the MIB-5 antibody was also found to be useful in monitoring cell proliferation in regenerative processes in tissues of adult animals.

The fact that MIB-5 is a mouse monoclonal antibody makes its application on murine tissue sections troublesome, because background staining due to endogenous immunoglobulins is difficult to avoid. To overcome this problem we first raised a rabbit antiserum against the murine Ki-67 protein equivalent (Kosco-Vilbois et al., 1997) and recently a rat monoclonal antibody was prepared in a collaboration between the laboratory of Hanswalter Zentgraf at the German Cancer Research Center (Heidelberg), our own group, and Dianova GmbH (Hamburg). This antibody, designated as TEC-3, was raised by immunizing rats with a recombinantly expressed part of the murine Ki-67 equivalent protein. Figure 1a shows an example for the staining obtained with this antibody on a murine tissue section. In general, the TEC-3 staining pattern is very similar to the Ki-67 staining pattern on the corresponding human tissues.

With the development of MIB-5 and TEC-3, a gap was closed. It is now possible to assess cell proliferation by the means of monoclonal Ki-67-equivalent antibodies in most mammals that are routinely used as experimental animals. This should lead to an increasing utilization of these antibodies in basic research. The possibility of identifying proliferative cells in mammalian tissues and cell cultures with little effort by immunological methods should be beneficial in various fields of basic and applied research. These include such different areas as drug screening, the study of animal models of various human diseases, wound healing and other regenerative processes, aging, and tumor biology as well as developmental biology. An interesting example of the latter is the recent paper of Lee et al. (1999). The authors demonstrated that the expression of the transcription factor whn is principally associated with terminally differentiated cells. Nevertheless, a small subclass of cells expresses whn in combination with the Ki-67 protein, suggesting that whn expression may be involved in the transition from the proliferative to the postproliferative state.

The utilization of the Ki-67 protein as a proliferation marker could be further extended if Ki-67 equivalent proteins in nonmammalian species could be identified. If these proteins exist, the characterization of homologues in *Xenopus*, *Drosophila*, and *Caenorhabditis* would be of special interest. The identification of these proteins may be valuable not only in assessing cell proliferation in these well-studied animal models but could even provide us with a clue to understanding the function of the mammalian Ki-67 protein.

THE BIOLOGY OF THE Ki-67 PROTEIN

From structure to function?

In 1993, 10 years after the initial description of the antigen, when the entire cDNA sequence coding for the Ki-67 protein was published by Schlüter et al., it could be expected that the identification of sequence motifs of potential functional significance and homology searches would lead to rapid progress in the elucidation of the function of the molecule; indeed, the primary structure revealed numerous interesting features. Two differentially spliced mRNA isoforms were described

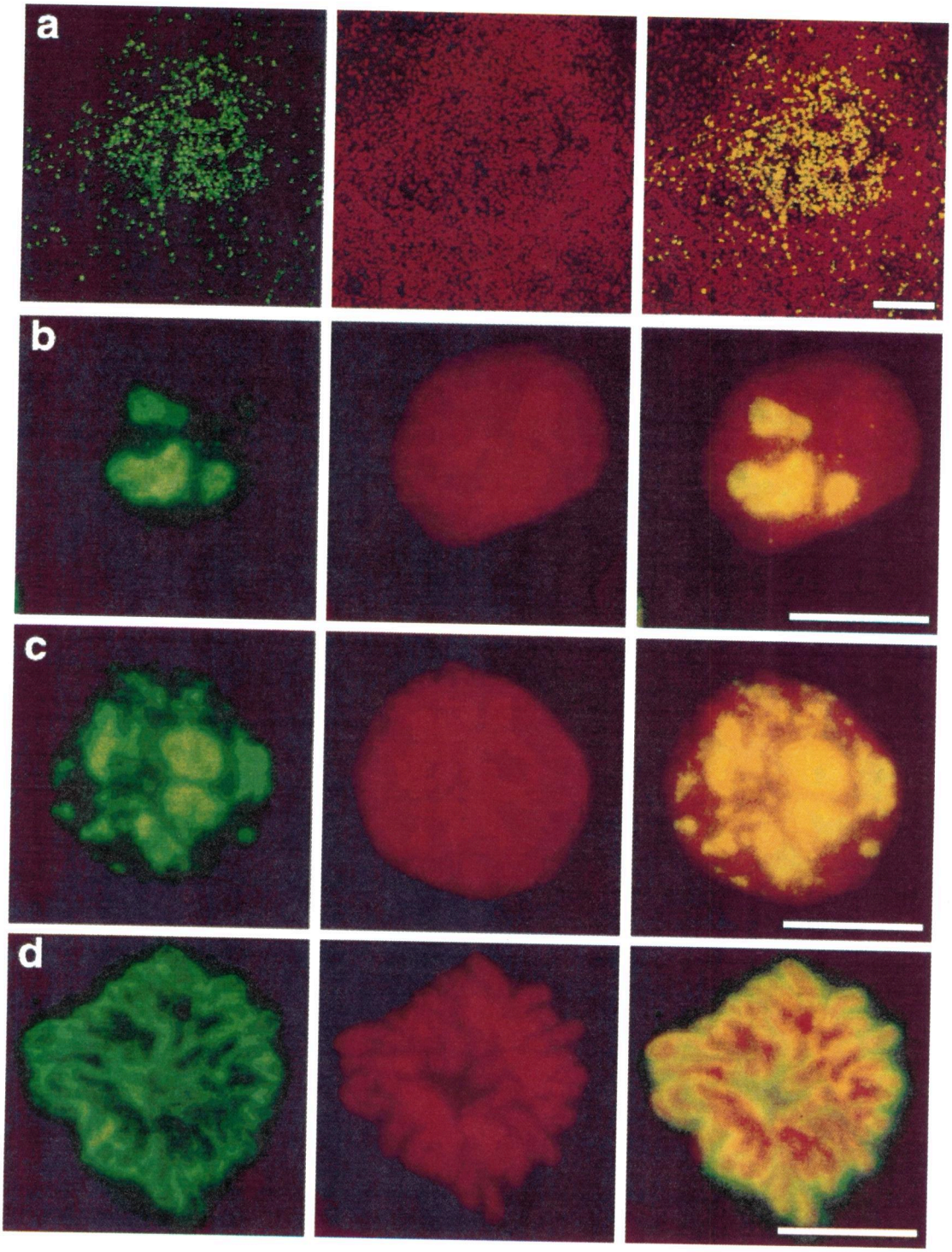

Fig. 1. Detection of the Ki-67 protein by immunofluorescence. Left-hand column, staining for the Ki-67 protein (green); center column, staining of DNA (red); right-hand column, merged images. (**a**) Murine lymph node stained with the rat monoclonal antibody TEC-3. Indirect immunofluorescence was performed using a fluorescein isothiocyanate-conjugated goat anti-rat IgG. Nuclei positive for the murine Ki-67 antigen appear in yellow. In the germinal centers, the nuclei of nearly all centroblasts and some centrocytes react with TEC-3. In contrast, most cells in the follicular mantle are negative for the Ki-67 antigen. Bar, 100 μm. (**b–d**) Cytospin preparations of human PHA stimulated peripheral mononuclear blood leukocytes stained with the monoclonal antibody MIB-1. Indirect immunofluorescence was performed using a fluorescein isothiocyanate-conjugated goat anti-mouse IgG. Bar, 10 μm. Note the different staining patterns during interphase (b, c) and mitosis (d).

with sizes of 8688 and 9768 base pairs. These two forms differ by the absence or presence of the region encoded by exon 7 of the Ki-67 gene. The molecular mass of the small and the large protein variant derived from these isoforms was calculated to be 320 and 359 kD, respectively. A potential "ATP/GTP binding site motif A" (P-loop) was predicted in the carboxy terminal region of the molecule (Saraste et al., 1990). Two putative nuclear targeting signals (Chelsky et al., 1989; Silver 1991) and eight potential "bipartite nuclear targeting signals" (Dingwall and Laskey, 1991) were in accordance with the finding that during interphase the antigen is exclusively detectable in the nuclei of the cells. Furthermore, the protein contains 143 protein kinase C, 89 casein kinase II, and 2 tyrosine kinase consensus sites.

Although some of the features predicted by the sequence analysis have been verified and characterized in later studies, for example, phosphorylation and nuclear transport (see later discussion), the function of the Ki-67 protein still remains unclear. In part, the difficulties in the determination of the functional role can be attributed to the absence of obvious homology with other proteins.

Although conserved domains, shared with proteins of characterized function would be an excellent guide in the initial steps of functional characterization, until recently (see later discussion), no protein—with the exception of some Ki-67 equivalents in other mammals—exhibits clear similarity with the human Ki-67 protein.

The failure to detect regions of similarity between Ki-67 and other proteins may be partially due to the fact that conventional search algorithms are too insensitive to detect only weakly conserved regions of homology. Using an advanced search method for sequence profiles, Hofmann and Bucher (1995) located a so-called forkhead-associated (FHA) domain in the amino terminal region of the Ki-67 protein. Interestingly, the authors could further show that this type of sequence motif is a common feature in a number of proteins known to be involved in cell-cycle regulation (e.g., DUN1 and RAD53 protein kinases from *S. cerevisiae* that link the S phase checkpoint to DNA-damage and the *S. pombe* cds1 kinase that is also involved in S phase regulation). Unfortunately, the function of the FHA domain is far from clear. Recently, it has been suggested that one of the two FHA domains of the Rad 53 protein kinase in *S. cerevisiae* is a protein binding domain that can interact with phosphorylated Rad9, thereby integrating DNA damage signals (Sun et al., 1998). Further progress in the understanding of the function of FHA domains and the identification of additional proteins that interact with these domains will lead to a better understanding of the cell cycle regulatory network and may also give some insight into the functional role of the Ki-67 protein.

A unique feature found in the Ki-67 primary structure is the occurrence of 16 repeated elements, each with a size of approximately 122 amino acid residues. These "Ki-67" repeats share between 43 and 62% identical amino acid residues. Within these repetitive elements there is a highly conserved region, also called the *Ki-67 motif*, exhibiting 72 to 100% identity with the consensus sequence. Moreover, the Ki-67 motif also includes the epitope (F K E L) that is recognized by the prototype Ki-67 antibody. The entire repetitive region is encoded by a single exon (13) of the Ki-67 gene. With a length of 6845 base pairs, Ki-67 exon 13 is one of the largest mammalian exons known.

Until recently, the Ki-67 repeats seemed to be a unique feature of the human Ki-67 protein and its equivalents in other mammals. This may have changed with the discovery of a new protein in cells from the rat kangaroo designated as *chmadrin* (Takagi et al., 1999). Like the Ki-67 protein, chmadrin contains a central repetitive domain. Although this domain is much smaller, the chmadrin repeats exhibit clear sequence similarity to the repeats of the Ki-67 protein. Similarity was also found between other limited regions of the proteins. Interestingly, like the Ki-67 protein, chmadrin contains an FHA domain close to its amino terminal end. Currently, it is not possible to decide whether chmadrin is the equivalent of the Ki-67 protein in the rat kangaroo or if it belongs to a new family of Ki-67-related proteins. In any case, the analysis of regions that are conserved in both proteins may be fruitful for the identification of functionally important domains and sequence motifs.

Ki-67 protein expression during the cell cycle

After preparation of the prototype Ki-67 antibody, it soon became evident that the recognized antigen could be exclusively detected in those cell populations that were known to proliferate (Gerdes et al., 1983). A detailed analysis of phytohemagglutinin (PHA) stimulated peripheral mononuclear blood leukocytes (PBL) showed that unstimulated (i.e., G_0 cells) were consistently negative for the Ki-67 antigen. In contrast, after PHA stimulation the antigen was expressed in S, G_2, and M phase cells (Gerdes et al., 1984). The expression in the first G_1 phase is controversially discussed. Whereas Lopez et al. (1991) claimed cells in the initial G_1 phase to be entirely negative for the Ki-67 antigen, Gerdes et al. (1984) reported the onset of Ki-67 expression already in the late G_1 phase (termed G_{1B}). However, both studies agree that G_1 cells in subsequent division cycles are positive for the Ki-67 protein, although there is controversy about the relative expression during the G_1 phase. Whereas some authors report an increase of Ki-67 antigen staining already starting in late G_1 phase (Braun et al., 1988; Starborg et al., 1996), others found a decrease until the onset of DNA synthesis (Lopez et al., 1991; Bruno and Darzynkiewicz, 1992). Van Dierendonck et al. (1989) even reported the striking observation that the Ki-67 antigen was undetectable in cells entering S phase, a fact that was disputed by others (du Manoir et al., 1991; Kill, 1996).

These inconsistencies can be explained, in part, by the hypothesis of du Manoir et al. (1991). The authors propose three different pathways during the G_1 phase. The *Ki-67 decrease pathway* is characterized by a declining Ki-67 staining and leads eventually to the exit from the active cell cycle (G_0). If cells on this pathway get stimulated by growth factors, they can enter the *Ki-67 increase pathway* that brings the cells back into S phase. Cells following the *Ki-67 stable pathway* exhibit a constant intensity of Ki-67 staining during the G_1 phase. This pathway is thought to correspond to optimal local growth conditions.

It is generally accepted that the Ki-67 antigen staining increases during S phase (du Manoir, 1991; Bruno and Darzynkiewicz, 1992). Moreover, it was shown that this even exceeds the increase in DNA content (Sasaki et al., 1987; Bruno et al., 1991). During G_2 phase a further increase in Ki-67 staining intensity was reported (du Manoir et al., 1991). The highest staining intensity of the Ki-67 antigen is found in metaphase (Braun et al., 1988; Verheijen et al., 1989b; du Manoir et al., 1991; Starborg et al., 1996), whereas during ana- and telophase the Ki-67 staining begins to decrease (Braun et al., 1988; Starborg et al., 1996).

These observations lead to the question in which way the changing staining intensity is linked to the biological function of the Ki-67 protein. The increase in Ki-67 staining intensity observed from the onset of S phase until metaphase cannot merely be explained by accumulation of the protein synthesized during this time period, because the biological half-life of the Ki-67 protein was estimated to be rather short. By radioactive pulse labeling of L428 cells, the half-life was determined to be approximately 90 min (Heidebrecht et al., 1996). This is in good agreement with the observation of Bruno and Darzynkiewicz (1992), who determined the decrease in Ki-67 staining intensity of HL60 after the inhibition of protein synthesis by cycloheximide. From these experiments the half-life of the Ki-67 protein was estimated to be approximately 1 h. Interestingly, the authors found no variance between cells in different cell cycle phases. The Ki-67 protein expression must therefore be the result of an exactly regulated de novo synthesis in association with effective degradation processes. This suggests that the precise regulation of the Ki-67 protein expression during the cell cycle may be crucial for its biological activity. As discussed in the next section, the cellular localization during the cell cycle seems to be of importance as well.

The cellular localization of the Ki-67 protein is cell cycle phase dependent

It is now well established that the cellular distribution of the Ki-67 protein is not constant but is subjected to dramatic changes during the cell division cycle (Fig. 1b,c,d). During the early G_1 phase, the Ki-67 staining is restricted to numerous foci throughout the nucleoplasm. It has been suggested that these foci correspond predominantly to the sites of the reforming nucleoli (van Dierendonck et al., 1989; du Manoir, 1991). In contrast, Kill (1996) found that although some of these foci are associated with sites containing nucleolar antigens, they are not necessarily co-localized. This was confirmed by a recent study showing that the Ki-67 protein is integrated into the reforming nucleoli at a relatively late time point, when fibrillarin, nucleolin, Nop52, and hPop1 are already present (Savino et al., 1999). In a detailed study, Bridger et al. (1998) showed that the localization of the Ki-67 protein in the very early G_1 phase coincides with regions of satellite DNA (centromeric alpha satellite, telomeric minisatellite, and the satellite III of heterochromatic blocks). During the progression of the G_1 phase, this co-localization declines. After integration of the Ki-67 protein into the nucleoli, only a few satellite regions remain associated with the Ki-67 staining. After reassembly of the nucleoli in the mid-G_1 phase, the Ki-67 protein is localized mainly in these structures (Braun et al., 1988; van Dierendonck et al., 1989; Verheijen, 1989a; Kill, 1996). For the murine Ki-67 equivalent, Starborg et al. (1996) showed that in addition to the nucleolar localization, the antigen could also be detected in the heterochromatic regions. Immunoelectron microscopy revealed that the Ki-67 protein is absent from the granular components and the fibrillar centers within the nucleoli (Verheijen, 1989a). It was suggested that the Ki-67 protein is confined to a newly defined compartment designated the "fibrillarin-deficient region of the dense fibrillar component" (Kill, 1996).

The distribution of the Ki-67 antigen during the S phase is a matter of discussion. According to Kill (1996), the Ki-67 staining during the S phase is restricted to the nucleoli, whereas other authors report an additional staining of the nucleoplasm (Braun et al., 1988; van Dierendonck et al., 1989; du Manoir et al., 1991). There are also controversial reports about the antigen distribution during the G_2 phase. Although some authors found the Ki-67 protein to be localized throughout the nucleus (du Manoir et al., 1991), others reported brightly stained foci in addition to a diffuse nucleoplasmic distribution (Braun et al., 1988). These foci, which only partially correspond to the nucleoli, have also been reported for the murine Ki-67 equivalent protein (Starborg et al., 1996).

A prominent redistribution of the Ki-67 protein occurs during mitosis. In prophase, the Ki-67 protein is reorganized and becomes detectable as a fine meshwork associated with the condensing chromatin (Verheijen et al., 1989b). In metaphase, a bright Ki-67 antigen staining is visible covering the surface of the individual chromosomes (Braun et al., 1988; Verheijen et al., 1989b; du Manoir et al., 1991; Starborg et al., 1996) (Fig. 1d). The entire Ki-67 protein, however, is not associated with the chromatin. After the breakdown of the nuclear membrane, part of the Ki-67 protein can also be detected distributed diffusely in the cytoplasm (Braun et al., 1988). Some authors reported that, toward the end of mitosis (ana- and telophase), the Ki-67 antigen exhibited a granular staining pattern (Braun et al., 1988; Starborg et al., 1996). In contrast, du Manoir et al. (1991) found a homogeneous staining during these phases.

The discrepancies described previously with regard to the localization of the Ki-67 protein during the cell cycle may be attributed, in part, to the fact that the studies were performed with different types of cells (even from different species), using different protocols for fixation and staining. An inherent problem with the visualization of the Ki-67 protein is the complete dependence on immunological methods for detection; however, it has been shown that the binding of antibodies to the Ki-67 protein depends on such factors as ionic strength (Bruno et al., 1992) or the binding of the Ki-67 protein to DNA (Lopez et al., 1994). On the one hand, this makes the visualization of the Ki-67 protein sensitive to the conditions used for fixation and staining of the samples. On the other hand, this leads to the intriguing speculation that the entire Ki-67 protein of the cell is not accessible to immunological detection with the antibodies available. It has been proposed that the newly synthesized Ki-67 in the cytoplasm cannot be detected, because the antigenic determinant is

masked (Braun et al., 1988). This may also be true in a more general way. The redistribution of the Ki-67 antigen staining described earlier may only in part be due to a genuine topological redistribution of the Ki-67 protein. The changing staining patterns may also reflect changes in the accessibility of the antigen, caused by alterations in the biological properties of the Ki-67 protein. Conformational changes, the forming of supramolecular complexes with other proteins, or the binding to nucleic acids may influence the interaction between antibody and antigen. It could be shown that in the presence of calcium ions, the Ki-67 protein structure is changed in such a way that the immunohistochemical detection with the monoclonal antibody MIB-1 was greatly reduced (Shi et al., 1999).

In this context, an alternative approach for the detection of the Ki-67 protein may prove to be useful. The expression of chimeric molecules consisting of the green fluorescent protein (GFP) and the Ki-67 protein (or parts thereof) by recombinant DNA technology may help to gain insight into the distribution of the Ki-67 protein during the cell cycle. A major advantage of this method is that these fusion proteins can be visualized in living cells without the need for fixation and subsequent antibody staining. Furthermore, by the expression of single protein domains or short fragments, it may be possible to identify the topogenic structures of the Ki-67 protein. A more detailed analysis of the cell-cycle-dependent localization, especially with respect to other nuclear macromolecules, may prove to be of crucial importance for insight into the functional role of the Ki-67 protein.

The biological significance of the Ki-67 protein

It is astounding that 16 years after its initial description, the functional role of the Ki-67 protein is still unknown. This is even more striking considering the enormous progress that has been made in the field of cell cycle research. So why has the Ki-67 protein never been linked to the network of proteins responsible for cell cycle control and progression? Could it be, that the Ki-67 protein, despite the strict correlation, is of no significance for cell proliferation? This is rather unlikely. It was shown that the incubation of cultured cells with oligodeoxynucleotides complementary to the Ki-67 mRNA inhibits DNA synthesis (Schlüter et al., 1993). Moreover, the microinjection of antibodies directed against the murine Ki-67 equivalent into the nuclei of Swiss-3T3 cells resulted in a decreased rate of cell division (Starborg et al., 1996). Similar results were obtained by microinjecting antibodies against the human Ki-67 protein (Heyden, 1997). In combination, these data strongly suggest that the Ki-67 protein has an essential role in cell proliferation.

As already mentioned, one reason for the paucity of knowledge regarding the Ki-67 protein function is the absence of apparent homology with other proteins of known function; other reasons are the size and the high susceptibility to protease cleavage. This makes the Ki-67 protein difficult to handle in biochemical assays, and with over 300 kD the molecule may easily be missed on standard gels for protein separation. Nevertheless, profound progress has recently been made in the characterization of the biological and biochemical properties. As described in the previous section, the cell-cycle-dependent reorganization of the Ki-67 protein has extensively been studied. It could been shown that some of these redistributions are accompanied by posttranslational modifications. For example, it was found that, during mitosis, the Ki-67 protein shifts to forms of higher apparent molecular mass and that these changes depend on protein phosphorylation (Elmer Endl, personal communication). Now it is necessary to determine which of the numerous theoretical predicted phosphorylation sites are of biological importance and which kinases and phosphatases are responsible for the modification of these sites.

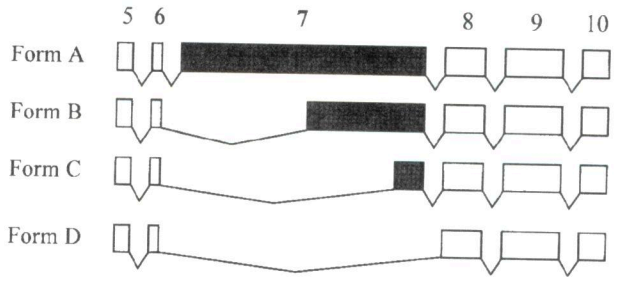

Fig. 2. Schematic representation of alternative splice forms of the Ki-67 mRNA found in murine tumor cell lines. Form A and Form D correspond to the "large" and the "small" isoform previously identified in human cells. Form B and Form C utilize internal splice acceptor sites located within the exon 7 equivalent. Exons and introns are indicated by boxes and lines, respectively.

Progress has also been made in the characterization of the signals that are responsible for the nuclear import of the Ki-67 protein. Although 10 putative nuclear targeting signals were predicted from the amino acid sequence (see previous discussion), one seems to be sufficient to accomplish nuclear import. When a carboxy terminal fragment of the Ki-67 protein, which contains only one putative "bipartite" nuclear targeting signal, is expressed in mammalian cells, this fragment alone, or as a GFP fusion protein, is targeted into the nucleus. In contrast, the predicted "bipartite" nuclear targeting signal located in the Ki-67 repeat 14 is not able to achieve nuclear localization (unpublished observation).

Another interesting finding is the presence of more than the two Ki-67 isoforms that were originally described. Analysis of the region encoded by exons 5–10 using reverse transcriptase PCR revealed multiple splice forms in mRNA preparations from human and murine cell lines. Figure 2 shows a schematic representation of the variants found in murine cells. In addition to the "large" and "small" forms, which correspond to the isoforms previously described in human cells (with or without exon 7), there are additional splice products that use acceptor sites located within the murine exon 7 equivalent. In all forms analyzed so far, the reading frame is kept intact, allowing for the generation of protein (Scholzen et al., 1997). In this context, it is tempting to speculate that the different splice variants are associated with certain "physiological states" of the cell, such as the positioning within the cell division cycle. This idea is supported by the observation that, in general, only one or two of these splice forms are detectable in single cells (Christiane Dimmler, personal communication).

As summarized in this study, the growing interest in the Ki-67 protein has led to an increasing number of publications dealing with different aspects of the biological properties of this molecule. In the near future these findings, like pieces of a jigsaw puzzle, may help to build a complete picture, providing an understanding of the biological significance of the Ki-67 protein, although at the moment, some of the central pieces are missing. How can these pieces be found? There are different strategies that may prove to be fruitful. The identification of binding partners, especially proteins, could be very helpful. In an initial step, co-localization studies may be useful in identifying candidates for the interaction with the Ki-67 protein. More direct approaches, like the two-hybrid-screening assay may also be productive.

Alternatively, the function of the Ki-67 protein could be inhibited. The functional role may then be deduced from the resulting phenotype. For example, functional inactivation could be achieved by the application of antibodies (see earlier discussion) or antisense molecules (DNA or RNA). Another promising approach is the generation of dominant-negative mutants (Herskowitz, 1987). The expression of such mutants inhibits the biological activity of the endogenous wild-type protein. A problem in the generation of these mutants lies in the fact that, without a detailed knowledge of protein function, it is difficult to predict which kind of mutant will exhibit a dominant-negative phenotype. Therefore, in the case of the Ki-67 protein, the generation of a dominant-negative mutant would be more or less a chance hit. Another way to inhibit protein function is the generation of null mutations in the mouse by gene targeting. The fact that the Ki-67 protein is probably essential for cell cycle progression makes it likely that the resulting phenotype is lethal. Therefore, a conditional approach, using the cre/loxP system (Gu et al., 1994; Rajewsky et al., 1996) may be the appropriate choice. In this system, a gene can be deleted only in certain cell populations of the organism (e.g., T cells or hepatocytes). This makes it possible to study null mutations, even when a complete "knock out" proves to be lethal for the organism, as it is to be expected for the Ki-67 protein.

Sixteen years after the initial description, we may now be on the verge of resolving the physiological role of the Ki-67 protein. It is to be hoped that the efforts to elucidate the biological function of this protein will not only contribute to improving our understanding of the complex regulatory network that governs cell proliferation, but also support the acceptance of the Ki-67 protein as a general proliferation marker.

ACKNOWLEDGMENTS

We are grateful to Mrs. Laura Haseley and Dr. Ulrike Seitzer for critical reading of the manuscript. Furthermore, we thank Dr. Christiane Gerlach and Dr. Elmar Endl for helpful discussions and review of the manuscript. We apologize to all colleagues whose work has not been cited due to space limitations.

LITERATURE CITED

Aaltomaa S, Lipponen P, Vesalainen S, Ala-Opas M, Eskelinen M, Syrjänen K. 1997. Value of Ki-67 immunolabelling as a prognostic factor in prostate cancer. Eur Urol 32:410–415.

Agarwal ML, Taylor WR, Chernov MV, Chernova OB, Stark GR. 1998. The p53 network. J Biol Chem 273:1–4.

Alison MR. 1995. Assessing cellular proliferation: what's worth measuring? Hum Exp Toxicol 14:935–944.

Archer SG, Eliopoulos A, Spandidos D, Barnes D, Ellis IO, Blamey RW, Nicholson RI, Robertson JF. 1995. Expression of ras p21, p53 and c-erbB-2 in advanced breast cancer and response to first line hormonal therapy. Br J Cancer 72:1259–1266.

Arellano M, Moreno S. 1997. Regulation of CDK/cyclin complexes during the cell cycle. Int J Biochem Cell Biol 29:559–573.

Bai XZ, Masters JR, O'Donoghue N, Kirby R, Pan LX, Young M, Stafford M, Parkinson MC. 1999. Prognostic markers in clinically localised prostate cancer. Int J Oncol 14:785–791.

Bettencourt M-C, Bauer JJ, Sesterhenn IA, Mostofi FK, McLeod DG, Moul JW. 1996. Ki-67 expression is a prognostic marker of prostate cancer recurrence after radical prostatectomy. J Urol 156:1064–1068.

Borre M, Bentzen SM, Nerstrøm B, Overgaard J. 1998. Tumor cell proliferation and survival in patients with prostate cancer followed expectantly. J Urol 159:1609–1614.

Braun N, Papadopoulos T, Müller-Hermelink HK. 1988. Cell cycle dependent distribution of the proliferation-associated Ki-67 antigen in human embryonic lung cells. Virchows Arch B Cell Pathol Incl Mol Pathol 56:25–33.

Bridger JM, Kill IR., Lichter P. 1998. Association of pKi-67 with satellite DNA of the human genome in early G_1 cells. Chromosome Res 6:13–24.

Bruno S, Darzynkiewicz Z. 1992. Cell cycle dependent expression and stability of the nuclear protein detected by Ki-67 antibody in HL-60 cells. Cell Prolif 25:31–40.

Bruno S, Crissman HA, Bauer KD, Darzynkiewicz Z. 1991. Changes in cell nuclei during S phase: progressive chromatin condensation and altered expression of the proliferation-associated nuclear proteins Ki-67, cyclin (PCNA), p105, and p34. Exp Cell Res 196:99–106.

Bruno S, Gorczyca W, Darzynkiewicz Z. 1992. Effect of ionic strength in immunocytochemical detection of the proliferation associated nuclear antigens p120, PCNA, and the protein reacting with Ki-67 antibody. Cytometry 13:496–501.

Bubendorf L, Sauter G, Moch H, Schmid HP, Gasser TC, Jordan P, Mihatsch MJ. 1996. Ki67 labelling index: an independent predictor of progression in prostate cancer treated by radical prostatectomy. J Pathol 178:437–441.

Bubendorf L, Tapia C, Gasser TC, Casella R, Grunder B, Moch H, Mihatsch MJ, Sauter G. 1998. Ki67 labeling index in core needle biopsies independently predicts tumor-specific survival in prostate cancer. Hum Pathol 29:949–954.

Cattoretti G, Becker MH, Key G, Duchrow M, Schlüter C, Galle J, Gerdes J. 1992. Monoclonal antibodies against recombinant parts of the Ki-67 antigen (MIB 1 and MIB 3) detect proliferating cells in microwave-processed formalin-fixed paraffin sections. J Pathol 168: 357–363.

Chelsky D, Ralph R, Jonak G. 1989. Sequence requirements for synthetic peptide-mediated translocation to the nucleus. Mol Cell Biol 9:2487–2492.

Cher ML, Stephenson RA, James BC, Carroll PR. 1996. Cellular proliferative fraction of metastatic lymph nodes predicts survival in stage D1 (TxN+M0) prostate cancer. J Urol 155:1674–1677.

Clahsen PC, van de Velde CJ, Duval C, Pallud C, Mandard A-M, Delobelle-Deroide A, van den Broek L, Sahmoud TM, van de Vijver MJ. 1998. p53 protein accumulation and response to adjuvant chemotherapy in premenopausal women with node-negative early breast cancer. J Clin Oncol 16:470–479.

Darzynkiewicz Z, Traganos F. 1982. RNA content and chromatin structure in cycling and noncycling cell populations studied by flow cytometry. In: Padilla P, McCarty KS, editors. Genetic expression in the cell cycle. New York: Academic Press. p 103–128.

Dettmar P, Harbeck N, Thomssen C, Pache L, Ziffer P, Fizi K, Jänicke F, Nathrath W, Schmitt M, Graeff H, Höfler H. 1997. Prognostic impact of proliferation-associated factors MIB1 (Ki-67) and S-phase in node-negative breast cancer. Br J Cancer 75:1525–1533.

Dingwall C, Laskey RA. 1991. Nuclear targeting sequences—a consensus? Trends Biochem Sci 16:478–481.

Domagala W, Markiewski M, Harezga B, Dukowicz A, Osborn M. 1996. Prognostic significance of tumor cell proliferation rate as determined by the MIB-1 antibody in breast carcinoma: its relationship with vimentin and p53 protein. Clin Cancer Res 2:147–154.

Drach J, Gattringer C, Glassl H, Drach D, Huber H. 1992. The biological and clinical significance of the KI-67 growth fraction in multiple myeloma. Hematol Oncol 10:125–134.

Drewinko B, Yang LY, Barlogie B, Trujillo JM. 1984. Cultured human

tumour cells may be arrested in all stages of the cycle during stationary phase: demonstration of quiescent cells in G_1, S and G_2 phase. Cell Tissue Kinet 17:453–463.

Duchrow M, Schlüter C, Wohlenberg C, Flad H-D, Gerdes J. 1996. Molecular characterization of the gene locus of the human cell proliferation-associated nuclear protein defined by monoclonal antibody Ki-67. Cell Prolif 29:1–12.

du Manoir S, Guillaud P, Camus E, Seigneurin D, Brugal G. 1991. Ki-67 labeling in postmitotic cells defines different Ki-67 pathways within the 2c compartment. Cytometry 12:455–463.

Evans T, Rosenthal ET, Youngblom J, Distel D, Hunt T. 1983. Cyclin: a protein specified by maternal mRNA in sea urchin eggs that is destroyed at each cleavage division. Cell 33:389–396.

Gerdes J, Schwab U, Lemke H, Stein H. 1983. Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation. Int J Cancer 31:13–20.

Gerdes J, Lemke H, Baisch H, Wacker H-H, Schwab U, Stein H. 1984. Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. J Immunol 133: 1710–1715.

Gerdes J, Li L, Schlueter C, Duchrow M, Wohlenberg C, Gerlach C, Stahmer I, Kloth S, Brandt E, Flad H-D. 1991. Immunobiochemical and molecular biologic characterization of the cell proliferation-associated nuclear antigen that is defined by monoclonal antibody Ki-67. Am J Pathol 138:867–873.

Gerlach C, Sakkab DY, Scholzen T, Daßler R, Alison MR, Gerdes J. 1997a. Ki-67 expression during rat liver regeneration after partial hepatectomy. Hepatology 26:573–578.

Gerlach C, Golding M, Larue L, Alison MR, Gerdes J. 1997b. Ki-67 immunoexpression is a robust marker of proliferative cells in the rat. Lab Invest 77:697–698.

Graña X and Reddy EP. 1995. Cell cycle control in mammalian cells: role of cyclins, cyclin-dependent kinases (CDKs), growth suppressor genes and cyclin-dependent kinase inhibitors (CKIs). Oncogene 11: 211–219.

Graña X, Garriga J, and Mayol X. 1998. Role of the retinoblastoma protein family, pRB, p107 and p130 in the negative control of cell growth. Oncogene 17:3365–3383.

Gu H, Marth JD, Orban PC, Mossmann H, Rajewsky K. 1994. Deletion of a DNA polymerase beta gene segment in T cells using cell type-specific gene targeting. Science 265:103–106.

Haerslev,T, Jacobsen GK, Zedeler K. 1996. Correlation of growth fraction by Ki-67 and proliferating cell nuclear antigen (PCNA) immunohistochemistry with histopathological parameters and prognosis in primary breast carcinomas. Breast Cancer Res Treat 37:101–113.

Heidebrecht HJ, Buck F, Haas K, Wacker HH, Parwaresch R. 1996. Monoclonal antibodies Ki-S3 and Ki-S5 yield new data on the "Ki-67" proteins. Cell Prolif 29:413–425.

Herskowitz I. 1987. Functional inactivation of genes by dominant negative mutations. Nature 329:219–222.

Heslin MJ, Cordon-Cardo C, Lewis JJ, Woodruff JM, Brennan MF. 1998. Ki-67 detected by MIB-1 predicts distant metastasis and tumor mortality in primary, high grade extremity soft tissue sarcoma. Cancer 83:490–497.

Heyden TS. 1997. Zellbiologische Untersuchungen zu intrazellulärem Transport, Funktion und Regulation des proliferations-assoziierten Antigens Ki-67. Doctoral thesis at the Freie Universität Berlin, Germany.

Hofmann K, Bucher P. 1995. The FHA domain: a putative nuclear signalling domain found in protein kinases and transcription factors. Trends Biochem Sci 20:347–349.

Huuhtanen RL, Blomqvist CP, Wiklund TA, Böhling TO, Virolainen MJ, Tukiainen EJ, Tribukait B, Andersson LC. 1999. Comparison of the Ki-67 score and S-phase fraction as prognostic variables in soft-tissue sarcoma. Br J Cancer 79:945–951.

Jansen RL, Hupperets PS, Arends JW, Joosten-Achjanie SR, Volovics A, Schouten HC, Hillen HF. 1998. MIB-1 labelling index is an independent prognostic marker in primary breast cancer. Br J Cancer 78:460–465.

Jensen V, Ladekarl M, Holm-Nielsen P, Melsen F, Sœrensen FB. 1995. The prognostic value of oncogenic antigen 519 (OA-519) expression and proliferative activity detected by antibody MIB-1 in node-negative breast cancer. J Pathol 176:343–352.

Kearsey SE, Labib K. 1998. MCM proteins: evolution, properties, and role in DNA replication. Biochim Biophys Acta 1398:113–136.

Keshgegian AA, Cnaan A. 1995. Proliferation markers in breast carcinoma. Mitotic figure count, S-phase fraction, proliferating cell nuclear antigen, Ki-67 and MIB-1. Am J Clin Pathol 104:42–49.

Keshgegian AA, Johnston E, Cnaan A. 1998. Bcl-2 oncoprotein positivity and high MIB-1 (Ki-67) proliferative rate are independent predictive markers for recurrence in prostate carcinoma. Am J Clin Pathol 110:443–449.

Kill IR. 1996. Localisation of the Ki-67 antigen within the nucleolus: evidence for a fibrillarin-deficient region of the dense fibrillar component. J Cell Sci 109:1253–1263.

Kosco-Vilbois MH, Zentgraf H, Gerdes J, Bonnefoy J-Y. 1997. To 'B' or not to 'B' a germinal center? Immunol Today 18:225–230.

Kubbutat MH, Cattoretti G, Gerdes J, Key G. 1994. Comparison of monoclonal antibodies PC10 and MIB 1 on microwave-processed paraffin sections. Cell Prolif 27:553–559.

Lazebnik YA, Medvedeva ND, Zenin VV. 1991. Reversible G2 block in the cell cycle of Ehrlich ascites carcinoma cells. Exp Cell Res 195: 247–254.

Lee D, Prowse DM, Brissette JL. 1999. Association between mouse nude gene expression and the initiation of epithelial terminal differentiation. Dev Biol 208:362–374.

Levine AJ. 1997. p53, the cellular gatekeeper for growth and division. Cell 88:323–331.

Lew DJ, Kornbluth S. 1996. Regulatory roles of cyclin dependent kinase phosphorylation in cell cycle control. Curr Opin Cell Biol 8:795–804.

Lloyd RV. 1998. Utility of Ki-67 as a prognostic marker in pancreatic endocrine neoplasms. Am J Clin Pathol 109:245–247.

Lopez F, Belloc F, Lacombe F, Dumain P, Reiffers J, Bernard P, Boisseau MR. 1991. Modalities of synthesis of Ki67 antigen during the stimulation of lymphocytes. Cytometry 12:42–49.

Lopez F, Belloc F, Lacombe F, Dumain P, Reiffers J, Bernard P, Boisseau MR. 1994. The labelling of proliferating cells by Ki67 and MIB-1 antibodies depends on the binding of a nuclear protein to the DNA. Exp Cell Res 210:145–153.

Mayol X and Graña X. 1997. pRB, p107 and p130 as transcriptional regulators: role in cell growth and differentiation. Prog Cell Cycle Res 3:157–169.

Miguel-Garcia A, Matutes E, Tarin F, Garcia-Talavera J, Miguel-Sosa A, Carbonell F, Catovsky D. 1995. Circulating Ki67 positive lymphocytes in multiple myeloma and benign monoclonal gammopathy. J Clin Pathol 48:835–839.

Molino A, Micciolo R, Turazza M, Bonetti F, Piubello Q, Bonetti A, Nortilli R, Pelosi G, Cetto GL. 1997. Ki-67 immunostaining in 322 primary breast cancers: associations with clinical and pathological variables and prognosis. Int J Cancer 74:433–437.

Moul JW, Bettencourt M-C, Sesterhenn IA, Mostofi FK, McLeod DG, Srivastava S, Bauer JJ. 1996. Protein expression of p53, bcl-2, and KI-67 (MIB-1) as prognostic biomarkers in patients with surgically treated, clinically localized prostate cancer. Surgery 120:159–166.

Paggi MG, Baldi A, Bonetto F, Giordano A. 1996. Retinoblastoma protein family in cell cycle and cancer: a review. J Cell Biochem 62:418–430.

Pinder,SE, Wencyk P, Sibbering DM, Bell JA, Elston CW, Nicholson R, Robertson JF, Blamey RW, Ellis IO. 1995. Assessment of the new proliferation marker MIB1 in breast carcinoma using image analysis: associations with other prognostic factors and survival. Br J Cancer 71:146–149.

Pines J. 1995. Cyclins and cyclin-dependent kinases: a biochemical view. Biochem J 308:697–711.

Prosperi E. 1997. Multiple roles of the proliferating cell nuclear antigen: DNA replication, repair and cell cycle control. Prog Cell Cycle Res 3:193–210.

Rajewsky K, Gu H, Kühn R, Betz UA, Müller W, Roes J, Schwenk F. 1996. Conditional gene targeting. J Clin Invest 98:600–603.

Rozan S, Vincent-Salomon A, Zafrani B, Validire P, De Cremoux P, Bernoux A, Nieruchalski M, Fourquet A, Clough K, Dieras V, Pouillart P, Sastre-Garau X. 1998. No significant predictive value of c-erbB-2 or p53 expression regarding sensitivity to primary chemotherapy or radiotherapy in breast cancer. Int J Cancer 79:27–33.

Rudolph P, Kellner U, Chassevent A, Collin F, Bonichon F, Parwaresch R, Coindre JM. 1997. Prognostic relevance of a novel proliferation marker, Ki-S11, for soft-tissue sarcoma: a multivariate study. Am J Pathol 150:1997–2007.

Rudolph P, Olsson H, Bonatz G, Ratjen V, Bolte H, Baldetorp B, Fernö M, Parwaresch R, Alm P. 1999. Correlation between p53, c-erbB-2, and topoisomerase IIa expression, DNA ploidy, hormonal receptor status and proliferation in 356 node-negative breast carcinomas: prognostic implications. J Pathol 187:207–216.

Saraste M, Sibbald PR, Wittinghofer A. 1990. The P-loop—a common motif in ATP- and GTP-binding proteins. Trends Biochem Sci 15: 430–434.

Sasaki K, Murakami T, Kawasaki M, Takahashi M. 1987. The cell cycle associated change of the Ki-67 reactive nuclear antigen expression. J Cell Physiol 133:579–584.

Savino TM, Bastos R, Jansen E, Hernandez-Verdun D. 1999. The

nucleolar antigen Nop52, the human homologue of the yeast ribosomal RNA processing RRP1, is recruited at late stages of nucleologenesis. J Cell Sci 112:1889–1900.
Sawhney N, Hall PA. 1992. Ki67—structure, function, and new antibodies. J Pathol 168:161–162.
Scalzo DA, Kallakury BV, Gaddipati RV, Sheehan CE, Keys HM, Savage D, Ross JS. 1998. Cell proliferation rate by MIB-1 immunohistochemistry predicts postradiation recurrence in prostatic adenocarcinomas. Am J Clin Pathol 109:163–168.
Schlüter C, Duchrow M, Wohlenberg C, Becker MH, Key G, Flad H-D, Gerdes J. 1993. The cell proliferation-associated antigen of antibody Ki-67: a very large, ubiquitous nuclear protein with numerous repeated elements, representing a new kind of cell cycle-maintaining proteins. J Cell Biol 123:513–522.
Scholzen T, Dimmler C, Wohlenberg C, Flad H-D, Gerdes J. 1997. Novel splice forms of the Ki-67 mRNA (Abstract). Eur J Cell Biol 72(Suppl. 44):40–40.
Schwarting R. 1993. Little missed markers and Ki-67. Lab Invest 68:597–599.
Seshadri R, Leong AS, McCaul K, Firgaira FA, Setlur V, Horsfall DJ. 1996. Relationship between p53 gene abnormalities and other tumour characteristics in breast-cancer prognosis. Int J Cancer 69: 135–141.
Shi S-R, Cote RJ, Hawes D, Thu S, Shi Y, Young LL, Taylor CR. 1999. Calcium-induced modification of protein conformation demonstrated by immunohistochemistry: what is the signal? J Histochem Cytochem 47:463–469.
Silver PA. 1991. How proteins enter the nucleus. Cell 64:489–497.
Starborg M, Gell K, Brundell E, Höög C. 1996. The murine Ki-67 cell proliferation antigen accumulates in the nucleolar and heterochromatic regions of interphase cells and at the periphery of the mitotic chromosomes in a process essential for cell cycle progression. J Cell Sci 109:143–153.
Stattin P, Damber J-E, Karlberg L, Bergh A. 1997. Cell proliferation assessed by Ki-67 immunoreactivity on formalin fixed tissues is a predictive factor for survival in prostate cancer. J Urol 157:219–222.
Stiegler P, Kasten M, Giordano A. 1998. The RB family of cell cycle regulatory factors. J Cell Biochem Suppl 30/31:30–36.
Sun Z, Hsiao J, Fay DS, Stern DF. 1998. Rad53 FHA domain associated with phosphorylated Rad9 in the DNA damage checkpoint. Science 281:272–274.
Tada S, Blow JJ. 1998. The replication licensing system. Biol Chem 379:941–949.
Takagi M, Matsuoka Y, Kurihara T, Yoneda Y. 1999. Chmadrin: a novel Ki-67 antigen-related perichromosomal protein possibly implicated in higher order chromatin structure. J Cell Sci 112:2463–2472.
Ueda T, Aozasa K, Tsujimoto M, Ohsawa M, Uchida A, Aoki Y, Ono K, Matsumoto K. 1989. Prognostic significance of Ki-67 reactivity in soft tissue sarcomas. Cancer 63:1607–1611.
van Dierendonck JH, KeijzerR, van de Velde CJ, Cornelisse CJ. 1989. Nuclear distribution of the Ki-67 antigen during the cell cycle: comparison with growth fraction in human breast cancer cells. Cancer Res 49:2999–3006.
van Oijen MG, Medema RH, Slootweg PJ, Rijksen G. 1998. Positivity of the proliferation marker Ki-67 in noncycling cells. Am J Clin Pathol 110:24–31.
Verheijen R, Kuijpers HJ, Schlingemann RO, Boehmer AL, van Driel R, Brakenhoff GJ, Ramaekers FC. 1989a. Ki-67 detects a nuclear matrix-associated proliferation-related antigen. I. Intracellular localization during interphase. J Cell Sci 92:123–130.
Verheijen R, Kuijpers HJ, van Driel R, Beck JL, van Dierendonck JH, Brakenhoff GJ, Ramaekers FC. 1989b. Ki-67 detects a nuclear matrix-associated proliferation-related antigen. II. Localization in mitotic cells and association with chromosomes. J Cell Sci 92:531–540.
Veronese SM, Maisano C, Scibilia J. 1996. Comparative prognostic value of Ki-67 and MIB-1 proliferation indices in breast cancer. Anticancer Res 16:2717–2722.
Wei W, Nurse P, Broek D. 1993. Yeast cells can enter a quiescent state through G1, S, G2, or M phase of the cell cycle. Cancer Res 53:1867–1870.
Weikel W, Brumm C, Wilkens C, Beck T, Knapstein PG. 1995. Growth fractions (Ki-67) in primary breast cancers, with particular reference to node-negative tumors. Cancer Detect Prev 19:446–450.

Bohn, Brent

From: Gibbons, Catherine
Sent: Friday, December 04, 2015 7:39 PM
To: Bohn, Brent
Subject: FW: Another Cr6 slide

From: Gibbons, Catherine
Sent: Tuesday, June 24, 2014 10:36 PM
To: Khan, Elaine@OEHHA <Elaine.Khan@oehha.ca.gov>
Subject: RE: Another Cr6 slide

Hey! Yeah still here, but I swear I won't pull an all nighter (again)! Thanks so much, I was so immersed in looking at all of this I didn't see your emails. I found the same thing in that 2013 paper, and normally I wouldn't have thought much of this had it not been for Chad's goofy graph with the up and down arrows. So hopefully I'll get a chance to ask him about this.

Also, puzzlingly, in that same paper they are talking about cytokine levels and saying these data support a pro-inflammatory response in rats and not in mice. WTF??

From: Khan, Elaine@OEHHA [mailto:Elaine.Khan@oehha.ca.gov]
Sent: Tuesday, June 24, 2014 7:43 PM
To: Gibbons, Catherine
Subject: RE: Another Cr6 slide

Ok, I just checked and slide 4 in this attachment (Science Question 4 presentation) refers to the mouse data (Thompson et al. 2013, Fig. 4). This is what they say in the paper:
"In addition, transcript levels of
Ki67, a common marker of crypt cell proliferation (Itzkovitz
et al., 2012; Potten et al., 1997), were significantly elevated at
_170 mg/l SDD at both day 8 and day 91 (Figure 4H).
Together, these data suggest that Cr(VI) increased crypt
hyperplasia as early as one week of exposure to very high
concentrations in drinking water."

To me, the decrease in Ki-67 at 91 days looks very similar to the their figure in slide 2 of their Science Question 3 presentation, where they say that "effects in rats might have been subsiding..." Attached is the Ki-67 review article I cited below – I haven't read it yet, but we may not need to read it since they themselves are saying that it's a marker of crypt cell proliferation.

From: Gibbons, Catherine [mailto:Gibbons.Catherine@epa.gov]
Sent: Tuesday, June 24, 2014 4:24 PM
To: Khan, Elaine@OEHHA
Subject: RE: Another Cr6 slide

LOL! As usual, turns out you have sent me this paper before and I forgot about it ☹ IN FACT, it was sitting RIGHT NEXT TO ME in a pile of "important papers to review" at this very second!

From: Khan, Elaine@OEHHA [mailto:Elaine.Khan@oehha.ca.gov]
Sent: Tuesday, June 24, 2014 7:16 PM

To: Gibbons, Catherine
Subject: RE: Another Cr6 slide

I notice slide 3 shows villous vacuolization – Chad had said before that they considered that part of the damage that led to crypt hyperplasia (because I noted that they had more animals with crypt hyperplasia than there were animals with apoptosis or villous atrophy) but I pointed out that vacuolization is not always a precursor of cell death. It's a stress response and can often lead to survival. See attached paper by Galluzzi et al. (highlights on p. 112 and Fig. 4 on p. 114).

Also, Ki-67 is a cell proliferation marker:

J Cell Physiol. 2000 Mar;182(3):311-22.

The Ki-67 protein: from the known and the unknown.

Scholzen T[1], Gerdes J.

Author information

- [1]Division of Molecular Immunology, Research Center Borstel, Germany. tscholzen@fz-borstel.de

Abstract

The expression of the human Ki-67 protein is strictly associated with cell proliferation. During interphase, the antigen can be exclusively detected within the nucleus, whereas in mitosis most of the protein is relocated to the surface of the chromosomes. The fact that the Ki-67 protein is present during all active phases of the cell cycle (G(1), S, G(2), and mitosis), but is absent from resting cells (G(0)), makes it an excellent marker for determining the so-called growth fraction of a given cell population. In the first part of this study, the term proliferation marker is discussed and examples of the applications of anti-Ki-67 protein antibodies in diagnostics of human tumors are given. The fraction of Ki-67-positive tumor cells (the Ki-67 labeling index) is often correlated with the clinical course of the disease. The best-studied examples in this context are carcinomas of the prostate and the breast. For these types of tumors, the prognostic value for survival and tumor recurrence has repeatedly been proven in uni- and multivariate analysis. The preparation of new monoclonal antibodies that react with the Ki-67 equivalent protein from rodents now extends the use of the Ki-67 protein as a proliferation marker to laboratory animals that are routinely used in basic research. The second part of this review focuses on the biology of the Ki-67 protein. Our current knowledge of the Ki-67 gene and protein structure, mRNA splicing, expression, and cellular localization during the cell-division cycle is summarized and discussed. Although the Ki-67 protein is well characterized on the molecular level and extensively used as a proliferation marker, the functional significance still remains unclear. There are indications, however, that Ki-67 protein expression is an absolute requirement for progression through the cell-division cycle.

So I'm not sure if that's mouse or rat data he's showing in slide 4, but if it's mouse, there shouldn't be a decrease in Ki-67 at 91 days compared to 8 days.

From: Gibbons, Catherine [mailto:Gibbons.Catherine@epa.gov]
Sent: Tuesday, June 24, 2014 3:14 PM
To: Khan, Elaine@OEHHA
Subject: Another Cr6 slide

Ha! Check this out, he's mentioning the paper and studies pointing to a villus origin for tumors.

Bohn, Brent

From: Gibbons, Catherine
Sent: Friday, December 04, 2015 7:38 PM
To: Bohn, Brent
Subject: FW: Cr6 meta-analysis
Attachments: Welling et al 2014 Cr+6.pdf

From: Khan, Elaine@OEHHA [mailto:Elaine.Khan@oehha.ca.gov]
Sent: Thursday, September 25, 2014 3:16 PM
To: Gibbons, Catherine <Gibbons.Catherine@epa.gov>; Sasso, Alan <Sasso.Alan@epa.gov>
Subject: Cr6 meta-analysis

OEM Online First, published on September 17, 2014 as 10.1136/oemed-2014-102178

Chromium VI and stomach cancer: a meta-analysis of the current epidemiological evidence

Roberta Welling,[1] James J Beaumont,[2] Scott J Petersen,[3] George V Alexeeff,[2] Craig Steinmaus[1]

▸ Additional material is published online only. To view please visit the journal online (http://dx.doi.org/10.1136/oemed-2014-102178).

[1]Office of Environmental Health Hazard Assessment, California Environmental Protection Agency, Oakland, California, USA
[2]Office of Environmental Health Hazard Assessment, California Environmental Protection Agency, Sacramento, California, USA
[3]Occupational Medicine, Kaiser Permanente, Oakland, California, USA

Correspondence to
Dr Craig Steinmaus, Office of Environmental Health Hazard Assessment, 1515 Clay Street, 16th Floor, Oakland, CA 94612 USA;
craigs@berkeley.edu

Received 13 March 2014
Revised 3 September 2014
Accepted 9 September 2014

ABSTRACT

Objectives Chromium VI (hexavalent chromium, Cr(VI)) is an established cause of lung cancer, but its association with gastrointestinal cancer is less clear. The goal of this study was to examine whether the current human epidemiological research on occupationally inhaled Cr(VI) supports the hypothesis that Cr(VI) is associated with human stomach cancer.

Methods Following a thorough literature search and review of individual studies, we used meta-analysis to summarise the current epidemiological literature on inhaled Cr(VI) and stomach cancer, explore major sources of heterogeneity, and assess other elements of causal inference.

Results We identified 56 cohort and case–control studies and 74 individual relative risk (RR) estimates on stomach cancer and Cr(VI) exposure or work in an occupation associated with high Cr(VI) exposure including chromium production, chrome plating, leather work and work with Portland cement. The summary RR for all studies combined was 1.27 (95% CI 1.18 to 1.38). In analyses limited to only those studies identifying increased risks of lung cancer, the summary RR for stomach cancer was higher (RR=1.41, 95% CI 1.18 to 1.69).

Conclusions Overall, these results suggest that Cr(VI) is a stomach carcinogen in humans, which is consistent with the tumour results reported in rodent studies.

What this paper adds

- Few studies have investigated the possible association between exposure to hexavalent chromium (Cr(VI)) and cancers other than respiratory cancers.
- This meta-analysis includes many more results than previous meta-analyses of Cr(VI) exposure and stomach cancer.
- Studies that were positive for lung cancer, which may indicate higher exposures, produced a higher summary relative risk for stomach cancer than the full meta-analysis.
- Possible mechanisms by which Cr(VI) might induce carcinogenesis are biologically plausible.

INTRODUCTION

Inhalation of hexavalent chromium (Cr(VI)) has occurred in a number of industries, including leather tanning, chrome plating, cement work and stainless steel welding and manufacturing. Numerous studies have identified associations between lung cancer and inhaled Cr(VI) in occupational settings, and the International Agency for Research on Cancer has classified Cr(VI) as a group I carcinogen, based primarily on studies of chromate production, chromate pigment production and chromium electroplating involving high exposures.[1] Given that the lung is directly exposed to inhaled Cr(VI), it is not surprising that this organ is a target site. However, several studies suggest that Cr(VI) may also have carcinogenic effects in other internal organs, including the gastrointestinal tract.

The issue of whether Cr(VI) causes gastrointestinal cancer has implications not only in exposed workers, but also in people who ingest Cr(VI) in drinking water. In a recent survey of 35 large US cities, Cr(VI) was detected in 89% of the water systems tested.[2] All levels were below the US Environmental Protection Agency's (US EPA) regulatory standard for chromium of 100 μg/L. However, this standard is based on a health risk assessment over 20 years old and is for total chromium (Cr(VI) and Cr(III) combined), not the more toxic Cr(VI). Based at least partially on its possible carcinogenicity in the gastrointestinal tract, US EPA and others are in the process of evaluating the need for a new Cr(VI) drinking water standard. To date, however, the evidence linking Cr(VI) to gastrointestinal cancer comes primarily from animal studies and questions have been raised about their relevance to humans. Our goal was to evaluate whether evidence from human studies supports the hypothesis that Cr(VI) is a cause of gastrointestinal cancer.

We performed a meta-analysis of human studies of Cr(VI) and stomach cancer in order to provide a review of the current literature, evaluate causal inference, and assess potential sources of bias and heterogeneity. Although we examined several types of gastrointestinal cancer, including oesophageal, small intestine and colon cancer, initial analyses showed that the greatest number of studies and clearest associations were seen for stomach cancer; thus, stomach cancer is the focus of this meta-analysis.

METHODS

Databases including Medline and EMBASE were searched by two authors independently (RW and CS) for all epidemiological studies on Cr(VI) and stomach cancer (ICD-9 code 151). Searches included combinations of the keywords or phrases: stomach, gastric, gastrointestinal, cancer, chromium, leather, tanning, stainless steel, cement,

To cite: Welling R, Beaumont JJ, Petersen SJ, *et al*. *Occup Environ Med* Published Online First: [*please include* Day Month Year] doi:10.1136/oemed-2014-102178

concrete, welding and metal plating. We also searched bibliographies of all publications included in the meta-analysis and all relevant review articles.

The meta-analysis included studies that provided relative risk (RR) estimates either specifically for Cr(VI) exposure or for workers in occupations known to be associated with Cr(VI) exposure, including chromate or chromium production and plating; leather work and tanning; Portland cement work; and stainless steel production, welding, polishing and grinding. Very few human studies have examined Cr(VI) in drinking water. Owing to this, and in order to maintain consistency by route of exposure, we excluded drinking water studies from the meta-analysis and review them in the discussion.

Only data published in peer-reviewed scientific journals were used, and government or industry reports were excluded. Studies of general foundry work and construction were also excluded because exposure is most likely low in many of these workers. Studies of asbestos cement workers and studies of shoe manufacturing, welding and metal plating that did not specifically evaluate chromium, stainless steel or leather workers were also excluded. Studies that reported no cases of stomach cancer were also excluded because of the inability to calculate a variance estimate, although this exclusion was evaluated in sensitivity analyses. In a few instances, a single paper reported separate RR estimates for men and women, or separate RR estimates for workers in different job categories or at different worksites. In these instances, we included all relative risks meeting our inclusion criteria when no clear overlap was present. We used Byar's approximation to estimate CIs in cohort studies in which they were not provided.[3] Each study was reviewed, and RR estimates and other information were abstracted independently by two authors (RW and CS).

Some studies gave RR estimates for several different metrics of Cr(VI) exposure, such as average exposure, peak exposure or exposure duration. In observational epidemiology, it is uncommon for all, or even most, studies to report findings using the same exposure metric. As a consequence, meta-analyses frequently involve combining data on different metrics. This meta-analysis is no different. When studies included RR estimates for different exposure metrics, we selected a single one in the following order: average exposure intensity, cumulative exposure and exposure duration. We chose this order a priori since analyses of other carcinogens have shown that exposure intensity may have a greater impact on cancer risks than exposure duration.[4 5] Several studies also reported relative risks for different levels of exposure (eg, high, medium, low). Since our goal was to evaluate whether an association exists, rather than defining exact dose–response relationships or exact low exposure risks, we selected the RR for the highest exposure category. If a true association exists, higher exposures will usually be associated with higher relative risks, and higher relative risks, all else being equal, have greater statistical power and are less likely to be due to bias or confounding than relative risks near 1.0.[6 7] The selected studies reported incidence rate ratios, ORs, standardised incidence ratios (SIRs) standardised mortality ratios (SMRs) or proportionate mortality ratios (PMRs). Some studies reported RR estimates adjusted for variables such as smoking, and these were used when available. For studies reporting data on incidence and mortality, incidence data were selected. Some studies reported results for different latency periods (ie, the time from first exposure to cancer diagnosis or death). Since many environmental agents can take decades to lead to detectable cancers, we chose the result for the longest latency, up to a maximum of 30+ years. For many cohort studies, publication of initial results was followed by updates, usually extending the period of follow-up. In these, we used the most recent publication giving the selected exposure metric or the largest number of cases. In a few publications of cement and leather work, Cr(VI) exposure was not specifically mentioned by the authors. These were included if the work processes described were those known to involve Cr(VI) exposure (eg, tanning or Portland cement). Inclusion and exclusion criteria are summarised in box 1.

In order to explore heterogeneity, we performed subgroup analyses on specific occupations, study design, incidence versus mortality, gender and country. Since it is possible that Cr(VI)

Box 1 Criteria for inclusion and exclusion of studies in the meta-analysis of Cr(VI) and stomach cancer

Inclusion criteria

- Epidemiological studies of stomach cancer and Cr(VI) exposure or work in an occupation known to be associated with Cr(VI) exposure including chromate or chromium production and plating; leather work and tanning; Portland cement work; and stainless steel production, welding, polishing and grinding
- Studies providing a relative risk estimate (including incidence rate ratios, ORs, standardised incidence ratios, standardised mortality ratios or proportionate mortality ratios) and the relative risk estimate's variance (or the data to calculate or estimate it)
- Published in peer-reviewed scientific journals
- If relative risk estimates are provided for different exposure metrics in a given study population, one metric was selected in the following order: average intensity, cumulative exposure, exposure duration
- If relative risk estimates are provided for different exposure levels in a given study population, the relative risk estimate for the highest level was selected
- Relative risk estimates adjusted for age, sex, smoking, diet and/or socioeconomic status were selected over unadjusted results
- If relative risk estimates for both stomach cancer mortality and incidence are reported in a given study population, the result for incidence was selected
- If relative risk estimates for different latency periods are reported in a given study population, the result for the longest latency period up to a period of 30+ years was selected
- For studies or relative risk estimates with overlapping populations, the most recent relative risk estimate with the selected exposure metric (eg, exposure intensity vs cumulative exposure; high vs low exposure level) or largest number of cases was selected

Exclusion criteria

- Unpublished data including government or industry reports
- Occupations such as painting, general foundry work, construction and shoe (non-leather) manufacturing
- Welding or metal plating studies that did not evaluate stainless steel or chromium work
- Studies involving work with asbestos cement
- Studies of all gastrointestinal cancers combined
- Studies of Cr(VI) in drinking water
- Studies reporting no cases of stomach cancer

exposures were too low in some studies to identify a true association, we conducted separate analyses of Cr(VI) and stomach cancer that included only studies in which elevated relative risks were identified for lung cancer, a well-established effect of high Cr(VI) exposure. In this analysis, since statistical significance is highly dependent on sample size (not just the presence of a true effect), we included all studies in which the RR of lung cancer was ≥1.5 regardless of statistical significance. Several subgroup and other analyses were done to evaluate potential confounding (eg, from smoking) and to compare our meta-analysis to other recent meta-analyses on this topic.

We calculated summary RR estimates using the fixed and random effects models.[8 9] We assessed heterogeneity among studies using the general variance-based method as described by Petitti.[10] Statistical heterogeneity was defined as present if the p value of the χ^2 test statistic was below 0.05. Some authors have suggested that because the random effects model incorporates between-study heterogeneity, it is more conservative than the fixed effects model.[10] However, a potential problem with the random effects model is that, unlike the fixed effects model, study weighting is not directly proportional to study precision. As a consequence, the random effects model gives relatively greater weight to smaller, less precise studies than the fixed effects model. This can sometimes lead to summary results that are less conservative than those produced using the fixed effects model.[11] To avoid this problem, we used the method presented by Shore *et al*[12] for our main results. In this method, the summary RR estimate is calculated by directly weighting individual studies by their precision, and between-study variability is only incorporated into calculations of variance (ie, the 95% CI). We assessed publication bias using funnel plots and Egger's and Begg's tests.[13 14] The funnel plot is a graphical presentation of each study's effect size versus an estimate of its precision. This plot can be asymmetric if smaller studies with results that are null or in the unexpected direction are not published. In Egger's test, asymmetry in the funnel plot is formally tested by performing a simple linear regression of the effect size divided by its SE on the inverse of the SE. In Begg's test, Kendall's rank order test is used to evaluate whether there is a correlation between the studies' effect sizes and their SEs. All calculations were performed using Microsoft Excel 2010 or STATA V.12 (College Station, Texas, USA) and all p values are two sided.

RESULTS

In total, 74 RR estimates, from 56 separate publications, met our inclusion criteria and were included in the meta-analysis (see online supplementalary table S1). Overall, 63 results (85%) were selected from cohort studies and 11 (15%) from case–control studies, and the meta-analysis involved studies that included 1399 cases of stomach cancer. Eighteen studies (24%) involved chromium production or plating, 23 (31%) involved cement workers, 17 (23%) involved leather work including tanning, four (5%) involved Cr(VI) or stainless steel welding, and 12 (16%) involved other occupations such as ferrochromium or other stainless steel work. Studies excluded from the meta-analysis and the reasons for their exclusion are shown in online supplementalary table S2.

The summary relative risk for all studies combined was 1.27 (95% CI 1.18 to 1.38; p<0.001; table 1). A forest plot summarising the results and weights applied to each study is shown in figure 1. Seventy per cent of the individual RR estimates in the overall analysis were >1.0. No single RR estimate received more than 14% of the total weight showing that no single study dominated the assigned weights. Summary relative risks were elevated for cement (1.29; 95% CI 1.17 to 1.42) and leather work (1.46; 95% CI 1.23 to 1.72) but not for welding (1.06; 95% CI 0.72 to 1.56). For studies of Cr(VI) production and plating, the summary RR was above 1.0 (1.25; 95% CI 0.97 to 1.60), but the 95% CI included 1.0. Summary relative risks were higher in case–control (1.55; 95% CI 1.16 to 2.07) than in cohort studies (1.26; 95% CI 1.16 to 1.37), males (1.30; 95% CI 1.20 to 1.41) than in females (1.08; 95% CI 0.65 to 1.81), and in studies of mortality (1.39; 95% CI 1.24 to 1.57) than in studies of incidence (1.17; 95% CI 1.07 to 1.29), but differences were only statistically significant when studies of incidence and mortality were compared (p=0.02). In the studies that identified Cr(VI)-associated lung cancer relative risks ≥1.5 (the proxy measure for probable higher exposure), the stomach cancer summary relative risk was 1.41 (95% CI 1.18 to 1.69; p<0.001) in all studies (figure 2) and 1.36 (95% CI 1.01 to 1.81; p=0.04) in Cr(VI) production and plating studies. The variables adjusted or stratified for in each study are shown in online supplementalary table S1. Only nine studies adjusted for some indicator of smoking, diet or socioeconomic status (SES), and the RR for these studies was 1.31 (1.01 to 1.69). Results in almost all analyses were similar regardless of whether the random effects model or the fixed effects model with the correction for between-study variability was used. For example, in the meta-analysis of all studies combined, the results using these two models were 1.28 (95% CI 1.16 to 1.41) and 1.27 (95% CI 1.18 to 1.38), respectively.

We saw no evidence of asymmetry in the funnel plot of all studies combined (figure 3), or in the funnel plots of each subgroup analysis (not shown). Egger's and Begg's tests also showed no consistent evidence of publication bias. For example, in the all studies combined analysis, the bias coefficient for Egger's test was 0.16 (p=0.55). In the analysis of all studies with lung cancer relative risks ≥1.5, the Egger's bias coefficient was 0.22 (p=0.64).

DISCUSSION

The overall summary relative risk of 1.27 (95% CI 1.18 to 1.38, p<0.001) provides evidence that Cr(VI) inhalation increases the risk of stomach cancer. The narrow CI, excluding 1.0, and the low p value provide evidence that this result is not due to chance. A major finding here is that the summary relative risk for stomach cancer was elevated in those studies in which Cr(VI)-associated lung cancer relative risks were also elevated, both in the analysis of all job categories combined (summary relative risk=1.41; 1.18 to 1.69; p<0.001) and in the analysis of chromium production and plating studies (summary relative risk=1.36; 1.01 to 1.81; p=0.04). Since Cr(VI) exposures, in general, are likely to be higher in those studies where increases in lung cancer were found, the presence of a positive lung cancer finding may be a valid surrogate for high Cr(VI) exposure. As such, these latter findings provide additional evidence that the positive findings seen in this meta-analysis are due to Cr(VI).

Statistically significant heterogeneity was seen in the meta-analysis of all studies combined (χ^2=139.6, p<0.001), and the CIs of several studies did not include the summary relative risk. However, we did not see statistically significant heterogeneity in most other analyses performed, including the analyses of studies with elevated lung cancer risks (χ^2=22.6, p=0.31). In observational epidemiology, study designs, populations, methods of assessing exposure and outcome, and statistical analyses are rarely, if ever, the same. As such, some variation across study results is expected. The fact that statistical heterogeneity

Table 1 Results of the meta-analysis of Cr(VI) exposure and stomach cancer

	No. of cases	No. of results*	Fixed effects model			Shore adjusted CI		Random effects model			Heterogeneity		
			RRs	CI_L	CI_U	CI_L	CI_U	RRs	CI_L	CI_U	χ^2	p Value	I^2 (%)
All studies	1399	74	1.27	1.20	1.35	1.18	1.38	1.28	1.16	1.41	139.6	<0.001	47.7
Job type													
Production or plating	113	18	1.25	1.02	1.53	0.97	1.60	1.25	0.95	1.65	25.9	0.08	34.4
Cement work	903	23	1.29	1.20	1.38	1.17	1.42	1.37	1.21	1.54	42.7	0.005	48.4
Leather work	237	17	1.46	1.27	1.67	1.23	1.72	1.33	1.08	1.64	23.6	0.10	32.1
Welding	31	4	1.06	0.72	1.55	0.72	1.56	1.08	0.72	1.56	3.0	0.39	0.8
All other	115	12	0.96	0.79	1.17	0.69	1.33	1.12	0.78	1.60	31.7	<0.001	65.3
Study design													
Case–control	130	11	1.55	1.16	2.07	NA	NA	NA	NA	NA	8.2	0.61	NA
Cohort	1269	63	1.26	1.19	1.34	1.16	1.37	1.25	1.13	1.39	129.6	<0.001	52.2
PMR studies	353	10	1.60	1.43	1.78	1.43	1.78	1.60	1.43	1.79	9.3	0.41	2.9
SMR studies	293	32	1.14	1.00	1.29	0.95	1.36	1.17	0.96	1.43	61.5	<0.001	49.6
Other	623	21	1.16	1.07	1.26	1.04	1.29	1.17	1.03	1.34	33.6	0.03	40.4
Incidence vs mortality													
Incidence studies	738	30	1.17	1.09	1.27	1.07	1.29	1.21	1.07	1.36	41.1	0.07	29.4
Mortality studies	661	44	1.39	1.28	1.51	1.24	1.57	1.32	1.14	1.53	89.8	<0.001	52.1
Gender													
Males only	1258	59	1.30	1.22	1.38	1.20	1.41	1.33	1.19	1.47	112.8	<0.001	48.6
Females only	23	6	1.08	0.72	1.63	0.65	1.81	1.14	0.61	2.11	8.0	0.16	37.4
Lung cancer RR ≥1.5													
All studies	170	21	1.41	1.19	1.67	1.18	1.69	1.41	1.16	1.71	22.6	0.31	11.4
Production or plating	78	13	1.36	1.06	1.73	1.01	1.81	1.31	0.96	1.80	16.9	0.15	29.0
Country, region													
Europe	859	48	1.16	1.08	1.25	1.06	1.27	1.20	1.06	1.35	78.2	0.003	39.9
North America	419	16	1.50	1.36	1.66	1.31	1.72	1.47	1.24	1.75	27.9	0.02	46.3
Asia	121	10	1.34	1.10	1.62	1.03	1.74	1.31	0.94	1.81	16.7	0.05	46.1

*Some publications provided two or more results that met the inclusion criteria but did not involve overlapping populations (eg, separate results for males and females).
CI_L, lower 95% CI; CI_U, upper 95% CI; I^2, the percentage of total variation across studies due to heterogeneity rather than chance; NA, not applicable (Shore adjusted CI (applied to the fixed effects RR) and the random effects model are only used when the χ^2 heterogeneity statistic is greater than the number of individual study results minus one); PMR, proportionate mortality ratio; RR, relative risk estimate; RRs, summary relative risk; SMR, standardised mortality ratio; χ^2, χ^2 heterogeneity statistic.

was not present in most of the subgroup analyses we performed highlights the overall consistency in many of these results. This consistency is supported by the fact that the large majority of individual RR estimates are >1.0. For example, in the analysis of all studies combined, 52 of 74 RR estimates are >1.0. The probability that this would occur by chance alone is 0.0002.

In this meta-analysis, as in almost all meta-analyses of epidemiological data, studies using different exposure metrics (eg, average exposure, exposure duration) were combined. The use of different metrics can potentially affect summary relative risks, but the likely direction is towards the null, not towards a false positive result. The reason for this is that if Cr(VI) is truly associated with stomach cancer, some metrics are likely to be more strongly associated with stomach cancer than others, and including less relevant metrics would dilute summary relative risks towards 1.0. If every study had reported data on the same single metric that was most strongly associated with stomach cancer, it is likely that the true summary relative risks would be even higher than those reported here. A similar effect could have resulted from our including studies with different levels of Cr (VI) exposure or different forms of Cr(VI). That is, if a true association exists, the inclusion of studies in which Cr(VI) exposures were relatively low would most likely bias results towards a summary relative risk of 1.0, not towards a false association. Previous research suggests that the absorption fraction is higher for soluble chromium compounds than for insoluble forms.[15] Few of the studies used in this meta-analysis provided details on Cr(VI) solubility. If less soluble forms are less carcinogenic, including studies involving these less soluble forms would dilute any associations due to soluble Cr(VI) to the null. It is most likely that all studies had at least some errors in assessing exposure. However, since they all assessed exposure using the same methods in people with and without cancer, this misclassification was most likely non-differential and also most likely biased findings towards the null.

Another factor that can potentially impact results is confounding. Most studies controlled for age and sex, but few adjusted for other factors (see online supplementalary table S1). The known risk factors for stomach cancer include older age; male sex; chronic gastritis and polyps; *Helicobacter pylori* infection, certain genetic abnormalities; lifestyle factors such as smoking, alcohol and diet (low fruit and vegetable intake or high intake of salted, smoked or nitrate-preserved foods); and coal mining, nickel refining, rubber and timber processing, and possibly exposure to asbestos.[16] Importantly, confounding factors must typically be associated with both Cr(VI) and stomach cancer, and these associations must be fairly strong to cause important confounding.[17] Some factors are most likely too rare (eg, genetic disorders, family history) or not associated strongly enough with Cr(VI) exposure (eg, *Helicobacter pylori*, a major risk factor for stomach cancer) to cause important confounding. Some cement products contain asbestos.[18] Although

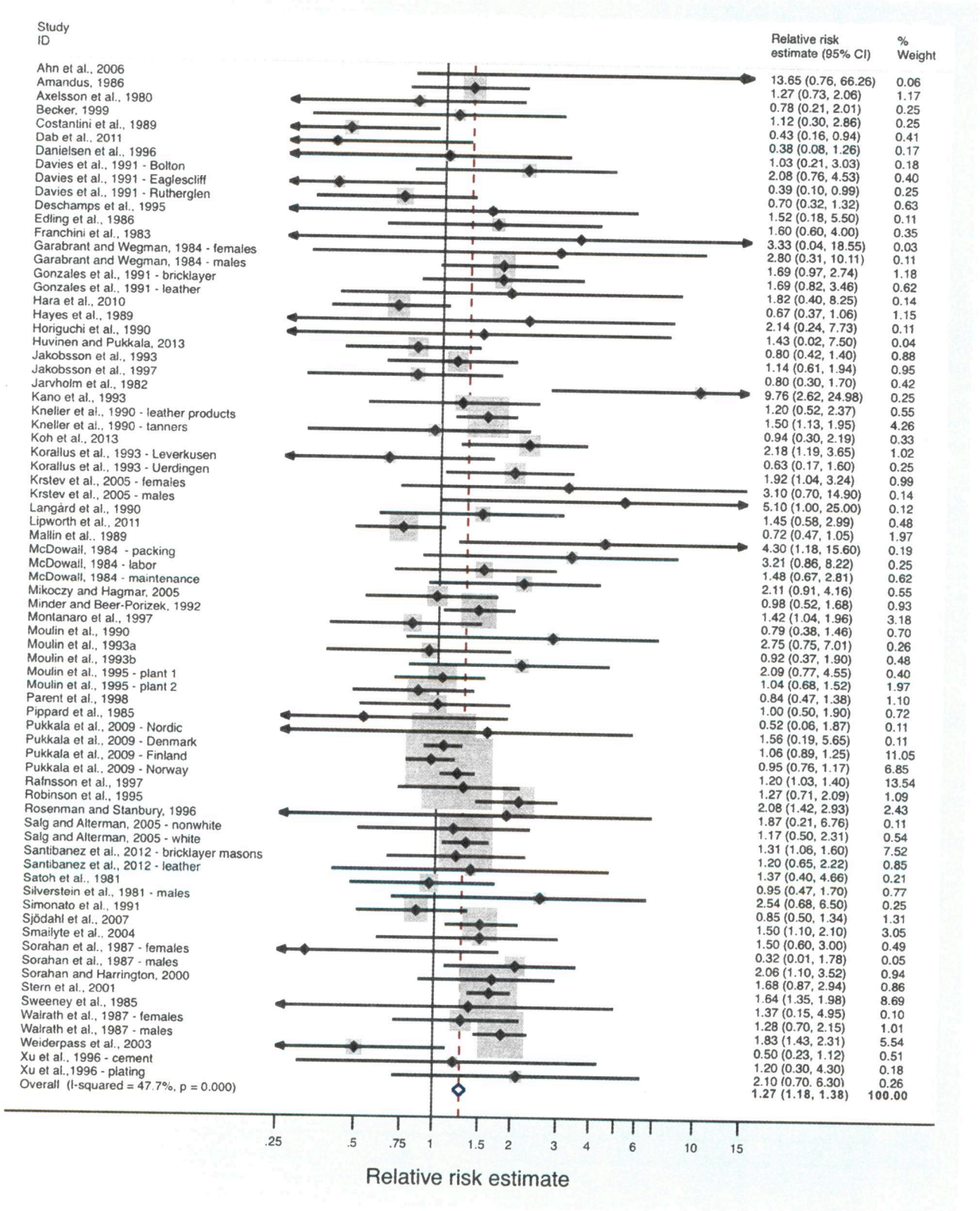

Figure 1 Forest plot of studies included in the meta-analysis of Cr(VI) and stomach cancer: all studies combined.

this could have potentially confounded results in cement workers, we excluded studies specifically in asbestos cement workers. In addition, high asbestos exposures were not known to have occurred in the other occupational categories assessed and summary relative risk estimates in cement workers were similar to those in several other job categories. A few studies adjusted for smoking, diet or SES, but the impacts of these adjustments are inconsistent, with an increase in relative risk

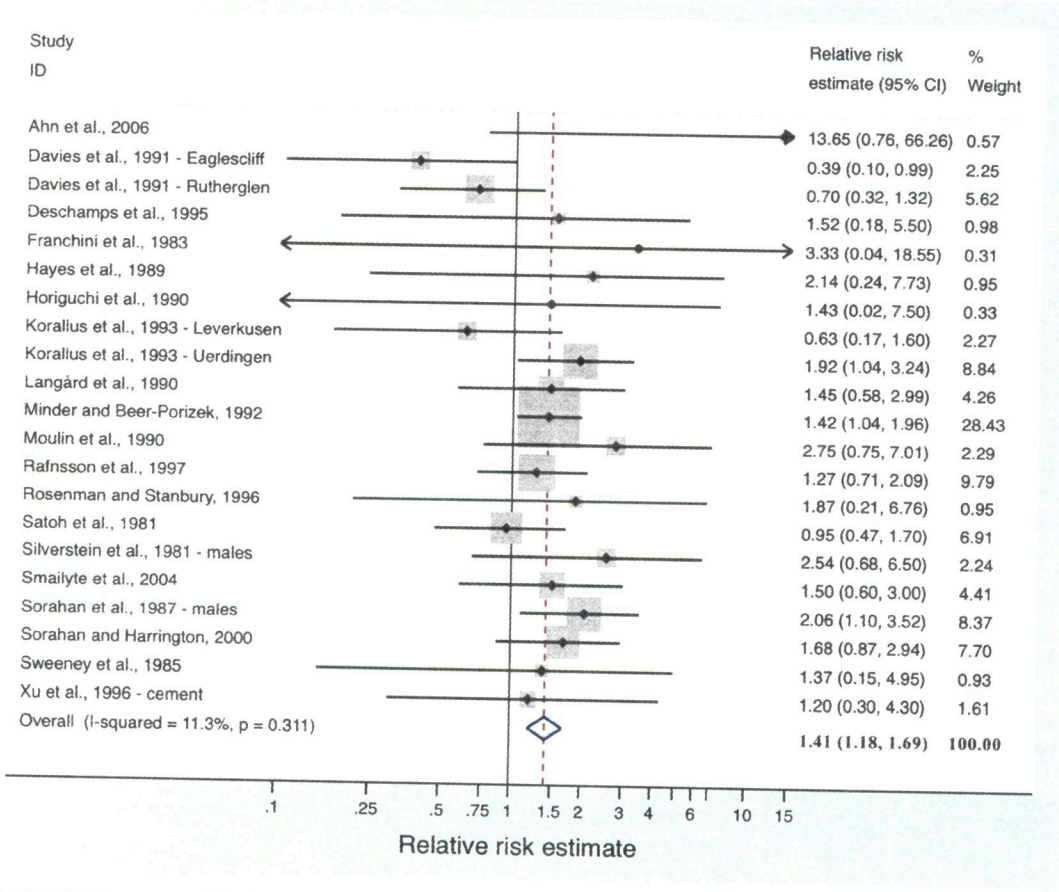

Figure 2 Forest plot of studies included in the meta-analysis of Cr(VI) and stomach cancer: only studies with lung cancer relative risk estimates ≥1.5.

estimates in some studies but a decrease in others. Axelson has shown that confounding by smoking may cause relative risks as high as 1.5 for lung cancer in occupational studies.[17] However, smoking-associated relative risks for stomach cancer are much lower than those for lung cancer, so the impact of smoking as a confounder is likely to be much less in studies of stomach cancer than in studies of lung cancer. Using the Axelson methods, and data on smoking-stomach cancer relative risks (about 1.5),[19] we estimated that confounding by smoking is unlikely to cause a relative risk >1.1 in occupational studies of stomach cancer.

The higher summary relative risks we identified for studies with positive lung cancer findings may indicate higher Cr(VI) exposure or it may indicate greater confounding by smoking. However, in a meta-analysis of those studies with lung cancer relative risk estimates ≥1.5 that provided data on non-malignant

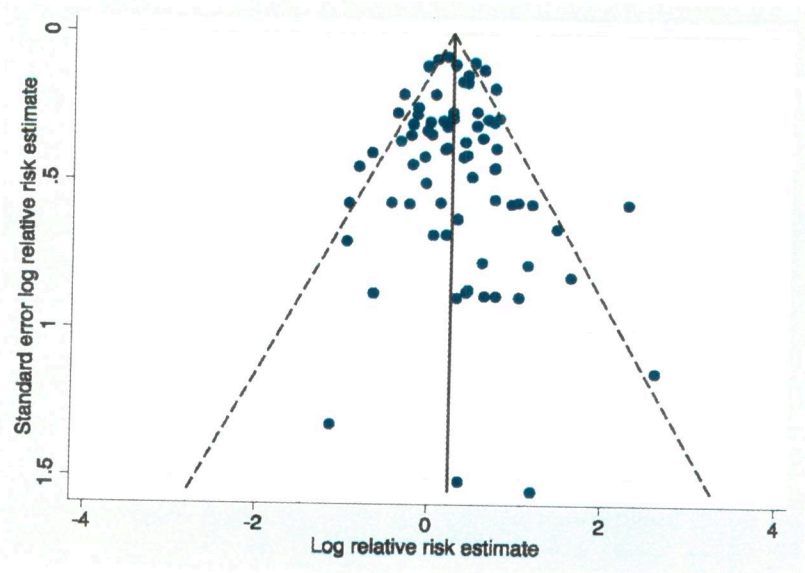

Figure 3 Funnel plot of studies included in the meta-analysis of Cr(VI) and stomach cancer: all studies combined.

respiratory disease (which is also caused by smoking), the summary RR for non-malignant respiratory disease was not elevated (RR=1.00; 95% CI 0.71 to 1.40; n=9; median relative risk estimate=0.91), providing evidence that smoking did not confound our results.

Other potential biases include the healthy worker effect and biases related to the inclusion of case-control studies (eg, recall bias or biased selection of controls). Although the summary relative risk for case-control studies was higher than that for cohort studies, the difference between these two was not statistically significant (p=0.18). The healthy worker effect would primarily affect studies comparing exposed workers to the general population (eg, SMRs) and this effect would most likely bias SMRs downwards. Although the extent of this bias here is unknown, evidence of the healthy worker effect has been reported for several different cancer types and in a number of different occupational settings.[20–22]

In this meta-analysis, neither visual inspection of the funnel plot nor Egger's or Begg's test showed evidence of publication bias, although the funnel plots are open to subjective interpretation, and Egger's and Begg's tests can be affected by factors other than this bias. Overall, while we did not see clear evidence of this bias, it is potentially an issue in any meta-analysis.

Two previous meta-analyses of Cr(VI) and stomach cancer have been published. In Gatto *et al*,[23] the summary relative risk involving 29 studies was 1.09 (95% CI 0.93 to 1.28). Similar to our meta-analysis, the Gatto *et al* meta-analysis included studies of chromium production, cement and leather workers (see online supplementalary table S3), but the individual study results are presented only in figure form, making direct comparisons with our meta-analysis difficult. One clear difference is our inclusion of many more results (74 vs 29), particularly from cement and leather workers, but also from studies of stainless steel and chromium plating workers. The summary relative risk using the individual RR estimates we abstracted for the 29 studies used by Gallo *et al* was somewhat lower than our meta-analysis of all 74 studies (1.22; 95% CI 1.05 to 1.41 vs 1.27; 95% CI 1.18 to 1.38). Another difference may have been our use of RR estimates from subgroups that are more likely to be highly exposed (eg, exposure duration ≥10 years), although direct comparisons are difficult for the reason given above. We also excluded five studies used by Gatto *et al* because they were unpublished, involved painters or foundry workers with uncertain exposure,[24 25] or overlapped with the already included studies.[26 27] However, adding these five excluded studies to our meta-analysis of all studies caused little change (1.27; 95% CI 1.18 to 1.37) since most of these studies only received a small amount of the total weighting. In a meta-analysis by Cole and Rodu, the authors reported that the summary relative risk between Cr(VI) and stomach cancer was lower in studies that adjusted for SES than in studies that did not adjust for this variable (RR=0.82 95% CI 0.69 to 0.96 vs RR=1.37; 95% CI 1.23 to 1.53), and concluded that SES was responsible for any apparent association seen between chromium exposure and stomach cancer.[28] However, one of the authors' criteria for these analyses was that studies "that were negative or essentially negative with respect to chrome exposure were included with the papers that were controlled [for SES]." In our evaluation of the studies used by these authors in their SES-controlled analysis, we were unable to find any mention of adjustments for SES (or any related variable) in 13 of the 14 studies (93%) included. Thus, the subgroup analysis titled 'SES-controlled' appears to be a misnomer, and instead reflects their criterion of studies that were 'negative or essentially negative with respect to chrome exposure.'

A variety of data support the biological plausibility of our results. Cr(VI) is a well-documented human lung carcinogen, and there is abundant evidence that airborne Cr(VI) is systemically absorbed. For example, studies in a variety of occupational settings have shown that Cr(VI) exposed workers have elevated blood or urine chromium levels compared to unexposed controls.[29 30] These data show that airborne Cr(VI) not only reaches the lungs, but that at least some of it is also internally absorbed and therefore most likely distributed to other organs. This systemic absorption may occur directly through the lungs, or particulates containing Cr(VI) that settle in the trachea and bronchi may be cleared by mucociliary action and then swallowed.[31] This swallowed Cr(VI) would come into direct contact with the stomach mucosa. Once in the stomach, ingested Cr(VI) is reduced by the acidic environment of the stomach to Cr(III), which is poorly absorbed. However, this reduction may not be complete, and most studies suggest that at least some ingested Cr(VI) escapes gastric reduction and is absorbed.[32] In studies in rodents, administration of Cr(VI) in drinking water has resulted in statistically significant increases in benign and malignant stomach tumours (combined),[31 33] papillomas or carcinomas (combined) of the oral cavity, and adenomas or carcinomas (combined) of the small intestine.[34] In humans, Beaumont *et al*[35] reported a RR of 1.82 (95% CI 1.11 to 2.91) for stomach cancer mortality in an area where Cr(VI) pollution from a ferrochromium factory caused widespread Cr(VI) contamination of nearby drinking water sources, although issues of dose–response and other potential biases have been debated.[36 37] In an ecological study in a province in Greece with Cr-contaminated water, SMRs were elevated for liver (SMR=11.0; 95% CI 4.05 to 24.0) and lung cancer (SMR=1.45; 95% CI 1.00 to 2.03).[38] The SMR for stomach cancer was above 1.0 but was not statistically significant (SMR=1.21; 95% CI 0.44 to 2.63).

The exact mechanisms by which Cr(VI) causes cancer are unknown, but evidence for several possible mechanisms exists. These include indirect and direct effects on DNA, epigenetic effects, gene regulation effects and direct cytotoxicity. Cr(VI) readily enters cells via active transport through anion channels and intracellular reduction follows, producing reactive intermediate Cr valences, Cr(V) and Cr(IV) and ultimately Cr(III), which is DNA-reactive. Reactive oxygen species, oxygen radicals and other reactive molecules generated during this reduction process are postulated to have genotoxic effects as well.[39–46] In vitro studies have revealed that Cr(VI)-induced mutations can be generated through different types of DNA damage such as interstrand crosslinks, DNA-protein crosslinks and DNA adducts, as well as single-strand and double-strand DNA breaks.[41 47 48] Studies of Cr(VI)-exposed tannery workers show evidence of genotoxic effects including chromosomal aberration, micronuclei formation, DNA breaks and higher levels of DNA damage in lymphocytes as determined by a comet assay.[49–52] In a study of chrome plating workers, chromium-induced DNA damage as measured by three comet assay components was significantly increased in exposed workers.[29] As a whole, these studies, along with the positive animal bioassays discussed above,[34] all provide biological plausibility for the findings of this meta-analysis.

CONCLUSIONS

The results of this meta-analysis suggest that Cr(VI) exposure is associated with increased risks of stomach cancer. An important feature of this study is that summary relative risks were elevated

in a number of different occupational settings and in the subgroup of studies in which lung cancer risks were also elevated. As with almost all meta-analyses, confounding and publication bias cannot be entirely ruled out. Few studies adjusted for some of the known risk factors of stomach cancer, including smoking, although an analysis of the potential magnitude of confounding from smoking suggests that this was unlikely to have caused the associations we observed. The exact relevance of our findings to Cr(VI) in drinking water is unknown. Differences in reduction and absorption patterns across the different routes of exposure could potentially impact toxicity. For example, the acidic environment of the stomach converts some ingested Cr(VI) to the poorly absorbed Cr(III), although several studies have shown that this process is not complete and some ingested Cr(VI) is absorbed.[53 54] Another difference is that drinking water exposures are generally much lower than occupational exposures, and this meta-analysis cannot be used to define exact dose–response relationships or low exposure risks. However, owing to the difficulties associated with studying lower exposures in human populations (a greater probability of bias, confounding and insufficient power),[6 37 55] chemical risk assessments and regulatory standards are frequently based on higher exposure occupational studies like the ones used here.[56] Another consideration is that drinking water exposures may cause greater toxicity because they can take place over the long term (eg, lifetime) and are more likely to occur at particularly susceptible life stages (eg, in fetuses, children and pregnant women) than exposures occurring at work. Thus, despite the different route and magnitude of exposure, our findings could have some relevance to efforts to regulate Cr(VI) in water in that they provide evidence that Cr(VI) is a cause of cancer in the human gastrointestinal tract and support the animal and limited human data linking ingested Cr(VI) to stomach cancer. US EPA and some states are considering regulating Cr(VI) in drinking water based on its potential carcinogenicity in the gastrointestinal tract, and California has recently established the first drinking water standard for Cr(VI) in the USA. The results of this study support such efforts.

Contributors CS, JJB, RW and GVA conceptualised the project and designed the overall study methods; CS and RW performed the literature searches and the statistical analyses; CS, JJB, RW, SJP and GVA assisted in the interpretation of results and writing.

Disclaimer The views expressed are those of the authors and do not necessarily represent those of the Office of Environmental Health Hazard Assessment, the California Environmental Protection Agency or the state of California.

Competing interests None.

Provenance and peer review Not commissioned; externally peer reviewed.

REFERENCES

1 Straif K, Benbrahim-Tallaa L, Baan R, *et al*. A review of human carcinogens—part C: metals, arsenic, dusts, and fibres. *Lancet Oncol* 2009;10:453–4.
2 Sutton R. *Chromium-6 in U.S. Tap Water*. Washington, DC: Environmental Working Group, 2010. http://www.ewg.org/chromium6-in-tap-water (accessed 23 Jul 2012).
3 Breslow NE, Day NE. *Statistical methods in cancer research. Vol. 2. The design and analysis of cohort studies*. Lyon: International Agency for Research on Cancer, 1987.
4 Lubin JH, Caporaso N, Wichmann HE, *et al*. Cigarette smoking and lung cancer: modeling effect modification of total exposure and intensity. *Epidemiology* 2007;18:639–48.
5 Lubin JH, Moore LE, Fraumeni JF Jr, *et al*. Respiratory cancer and inhaled inorganic arsenic in copper smelters workers: a linear relationship with cumulative exposure that increases with concentration. *Environ Health Perspect* 2008;116:1661–5.
6 Hill AB. The environment and disease: association or causation? *Proc R Soc Med* 1965;58:295–300.
7 Rothman K, Greenland S. Causation and causal inference. In: Rothman K, Greenland S. eds. *Modern epidemiology*. 2nd edn. Philadelphia: Lippincott Raven, 1998:7–28.
8 DerSimonian R, Laird N. Meta-analysis in clinical trials. *Control Clin Trials* 1986;8:177–88.
9 Greenland S. Quantitative methods in the review of epidemiologic literature. *Epidemiol Rev* 1987;9:1–30.
10 Petitti D. *Meta-analysis, decision analysis, and cost effective analysis: methods for quantitative synthesis in medicine*. New York: Oxford University Press, 1994.
11 Poole C, Greenland S. Random-effects meta-analyses are not always conservative. *Am J Epidemiol* 1999;150:469–75.
12 Shore R, Gardner M, Pannett B. Ethylene oxide: an assessment of the epidemiological evidence on carcinogenicity. *Br J Ind Med* 1993;50:971–97.
13 Begg CB, Mazumdar M. Operating characteristics of a rank correlation test for publication bias. *Biometrics* 1994;50:1088–101.
14 Egger M, Davey Smith G, Schneider M, *et al*. Bias in meta-analysis detected by a simple, graphical test. *BMJ* 1997;315:629–34.
15 Agency for Toxic Substances and Disease Registry (ATSDR). *Toxicological profile for Chromium*. Atlanta, GA: U.S. Department of Health and Human Services, Public Health Service, 2012.
16 Brenner H, Rothenbacher D, Arndt V. Epidemiology of stomach cancer. *Methods Mol Biol* 2009;472:467–77.
17 Axelson O. Aspects on confounding in occupational health epidemiology. *Scand J Work Environ Health* 1978;4:98–102.
18 Williams PR, Phelka AD, Paustenbach DJ. A review of historical exposures to asbestos among skilled craftsmen (1940–2006). *J Toxicol Environ Health B Crit Rev* 2007;10:319–77.
19 Ladeiras-Lopes R, Pereira AK, Nogueira A, *et al*. Smoking and gastric cancer: systematic review and meta-analysis of cohort studies. *Cancer Causes Control* 2008;19:689–701.
20 Kirkeleit J, Riise T, Bjorge T, *et al*. The healthy worker effect in cancer incidence studies. *Am J Epidemiol* 2013;177:1218–24.
21 Pukkala E, Martinsen JI, Lynge E, *et al*. Occupation and cancer—follow-up of 15 million people in five Nordic countries. *Acta Oncol* 2009;48:646–790.
22 Steinmaus C, Smith AH, Jones RM, *et al*. Meta-analysis of benzene exposure and non-Hodgkin lymphoma: biases could mask an important association. *Occup Environ Med* 2008;65:371–8.
23 Gatto NM, Kelsh MA, Mai DH, *et al*. Occupational exposure to hexavalent chromium and cancers of the gastrointestinal tract: a meta-analysis. *Cancer Epidemiol* 2010;34:388–99.
24 Guberan E, Usel M, Raymond L, *et al*. Disability, mortality, and incidence of cancer among Geneva painters and electricians: a historical prospective study. *Br J Ind Med* 1989;46:16–23.
25 Sorahan T, Faux AM, Cooke MA. Mortality among a cohort of United Kingdom steel foundry workers with special reference to cancers of the stomach and lung, 1946–90. *Occup Environ Med* 1994;51:316–22.
26 Costantini AS, Paci E, Miligi L, *et al*. Cancer mortality among workers in the Tuscan tanning industry. *Br J Ind Med* 1989;46:384–8.
27 Iaia TE, Bartoli D, Calzoni P, *et al*. A cohort mortality study of leather tanners in Tuscany, Italy. *Am J Ind Med* 2006;49:452–9.
28 Cole P, Rodu B. Epidemiologic studies of chrome and cancer mortality: a series of meta-analyses. *Regul Toxicol Pharmacol* 2005;43:225–31.
29 Danadevi K, Rozati R, Banu BS, *et al*. Genotoxic evaluation of welders occupationally exposed to chromium and nickel using the Comet and micronucleus assays. *Mutagenesis* 2004;19:35–41.
30 Gambelunghe A, Piccinini R, Ambrogi M, *et al*. Primary DNA damage in chrome-plating workers. *Toxicology* 2003;188:187–95.
31 Sedman RM, Beaumont J, McDonald TA, *et al*. Review of the evidence regarding the carcinogenicity of hexavalent chromium in drinking water. *J Environ Sci Health C Environ Carcinog Ecotoxicol Rev* 2006;24:155–82.
32 Donaldson RM Jr, Barreras RF. Intestinal absorption of trace quantities of chromium. *J Lab Clin Med*. 1966;68:484–93.
33 Borneff J, Engelhardt K, Griem W, *et al*. [Carcinogens in water and soil. XXII. Experiment with 3,4-benzopyrene and potassium chromate in mice drink]. *Arch Hyg Bakteriol* 1968;152:45–53.
34 NTP. *Technical Report on the Toxicology and Carcinogenesis Studies of Sodium Dichromate Dihydrate in F344/N Rats and B6C3F1 Mice. NTP TR 546*. NIH Publication No. 07-5887. Research Triangle Park, North Carolina: National Toxicology Program, 2008. http://ntp.niehs.nih.gov/ntp/htdocs/LT_rpts/tr546.pdf (accessed 10 Mar 2011).
35 Beaumont JJ, Sedman RM, Reynolds SD, *et al*. Cancer mortality in a Chinese population exposed to hexavalent chromium in drinking water. *Epidemiology* 2008;19:12–23.
36 Paustenbach DJ. On the chromium reanalysis. *Epidemiology* 2009;20:625–6; author reply 6–7.
37 Smith AH. Hexavalent chromium, yellow water, and cancer: a convoluted saga. *Epidemiology* 2008;19:24–6.
38 Linos A, Petralias A, Christophi CA, *et al*. Oral ingestion of hexavalent chromium through drinking water and cancer mortality in an industrial area of Greece—an ecological study. *Environ Health* 2011;10:50.
39 Benova D, Hadjidekova V, Hristova R, *et al*. Cytogenetic effects of hexavalent chromium in Bulgarian chromium platers. *Mutat Res* 2002;514:29–38.

40 Kasprzak KS. Possible role of oxidative damage in metal-induced carcinogenesis. *Cancer Invest* 1995;13:411–30.
41 O'Brien TJ, Ceryak S, Patierno SR. Complexities of chromium carcinogenesis: role of cellular response, repair and recovery mechanisms. *Mutat Res* 2003;533:3–36.
42 Shi X, Chiu A, Chen CT, *et al*. Reduction of chromium(VI) and its relationship to carcinogenesis. *J Toxicol Environ Health B Crit Rev* 1999;2:87–104.
43 Sugden KD, Burris RB, Rogers SJ. An oxygen dependence in chromium mutagenesis. *Mutat Res* 1990;244:239–44.
44 Sugiyama M. Role of physiological antioxidants in chromium(VI)-induced cellular injury. *Free Radic Biol Med* 1992;12:397–407.
45 Vaglenov A, Nosko M, Georgieva R, *et al*. Genotoxicity and radioresistance in electroplating workers exposed to chromium. *Mutat Res* 1999;446:23–34.
46 Witt KL, Stout MD, Herbert RA, *et al*. Mechanistic insights from the NTP studies of chromium. *Toxicol Pathol* 2013;41:326–42.
47 Jomova K, Valko M. Advances in metal-induced oxidative stress and human disease. *Toxicology* 2011;283:65–87.
48 McCarroll N, Keshava N, Chen J, *et al*. An evaluation of the mode of action framework for mutagenic carcinogens case study II: chromium (VI). *Environ Mol Mutagen* 2010;51:89–111.
49 Balachandar V, Arun M, Mohana Devi S, *et al*. Evaluation of the genetic alterations in direct and indirect exposures of hexavalent chromium [Cr(VI)] in leather tanning industry workers North Arcot District, South India. *Int Arch Occup Environ Health* 2010;83:791–801.
50 Medeiros MG, Rodrigues AS, Batoreu MC, *et al*. Elevated levels of DNA-protein crosslinks and micronuclei in peripheral lymphocytes of tannery workers exposed to trivalent chromium. *Mutagenesis* 2003;18:19–24.
51 Sbrana I, Caretto S, Lascialfari D, *et al*. Chromosomal monitoring of chromium-exposed workers. *Mutat Res* 1990;242:305–12.
52 Zhang M, Chen Z, Chen Q, *et al*. Investigating DNA damage in tannery workers occupationally exposed to trivalent chromium using comet assay. *Mutat Res* 2008;654:45–51.
53 Finley BL, Kerger BD, Katona MW, *et al*. Human ingestion of chromium (VI) in drinking water: pharmacokinetics following repeated exposure. *Toxicol Appl Pharmacol* 1997;142:151–9.
54 Kerger BD, Paustenbach DJ, Corbett GE, *et al*. Absorption and elimination of trivalent and hexavalent chromium in humans following ingestion of a bolus dose in drinking water. *Toxicol Appl Pharmacol* 1996;141:145–58.
55 Gibb H, Haver C, Gaylor D, *et al*. Utility of recent studies to assess the National Research Council 2001 estimates of cancer risk from ingested arsenic. *Environ Health Perspect* 2011;119:284–90.
56 U.S. Environmental Protection Agency. Integrated Risk Information System. 2013. http://www.epa.gov/IRIS/ (accessed 10 Jul 2013).

Chromium VI and stomach cancer: a meta-analysis of the current epidemiological evidence

Roberta Welling, James J Beaumont, Scott J Petersen, et al.

Occup Environ Med published online September 17, 2014
doi: 10.1136/oemed-2014-102178

Bohn, Brent

From: Gibbons, Catherine
Sent: Friday, December 04, 2015 7:38 PM
To: Bohn, Brent
Subject: FW: revised Cr6 reduction model published

From: Khan, Elaine@OEHHA [mailto:Elaine.Khan@oehha.ca.gov]
Sent: Tuesday, August 26, 2014 9:54 PM
To: Sasso, Alan <Sasso.Alan@epa.gov>; Wong, Patty@OEHHA <Patty.Wong@oehha.ca.gov>
Cc: Gibbons, Catherine <Gibbons.Catherine@epa.gov>
Subject: RE: revised Cr6 reduction model published

Congratulations, Alan! Looking forward to reading it!

Elaine

From: Sasso, Alan [Sasso.Alan@epa.gov]
Sent: Tuesday, August 26, 2014 6:08 AM
To: Wong, Patty@OEHHA
Cc: Gibbons, Catherine; Khan, Elaine@OEHHA
Subject: revised Cr6 reduction model published

Hi Elaine and Patty,

Some of the toxicokinetic work we did here at EPA has been published. It is currently in press in Toxicology and applied pharmacology:

http://www.sciencedirect.com/science/article/pii/S0041008X14003020

It will be an open-access article. The paperwork hasn't yet gone through for open access (may take a month), so you'll need a subscription to the journal at the moment.

-Alan

Alan F. Sasso, Ph.D.
Office of Research and Development
National Center for Environmental Assessment
U.S. Environmental Protection Agency
(703)-347-0179
